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<p>(21) International Application Number: <b>PCT/EP96/00080</b> (22) International Filing Date: 3 January 1996 (03.01.96) (30) Priority Data: 95200008.1 4 January 1995 (04.01.95) EP (34) Countries for which the regional or international application was filed: NL et al. 95202415.6 7 September 1995 (07.09.95) EP (34) Countries for which the regional or international application was filed: NL et al. (71) Applicant (for all designated States except US): <b>MOGEN INTERNATIONAL N.V. [NL/NL]; Einsteinweg 97, NL-2333 CB Leiden (NL).</b> (72) Inventors; and (75) Inventors/Applicants (for US only): <b>GODDIJN, Oscar, Johannes, Maria [NL/NL]; Oude Herengracht 5, NL-2312 LN Leiden (NL). VERWOERD, Teunis, Cornelis [NL/NL]; Oude Singel 268, NL-2312 RK Leiden (NL). KRUTWAGEN, Ronny, Wilhelmus, Hermanus, Henrika [NL/NL]; Concordiastraat 20, NL-2406 XZ Alphen aan den Rijn (NL). VOOGD, Eline [NL/NL]; Milanenhorst 26, NL-2317 CG Leiden (NL).</b></p>		<p>(74) Agent: <b>VAN WEZENBEEK, Lambertus, A., C., M.; Mogen International N.V., Einsteinweg 97, NL-2333 CB Leiden (NL).</b> (81) Designated States: <b>AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b>  <b>Published</b> <i>With international search report.</i> <i>With an indication in relation to a deposited microorganism furnished under Rule 13bis separately from the description.</i> <i>Date of receipt by the International Bureau:</i> <b>7 February 1996 (07.02.96)</b></p>
<p>(54) Title: <b>ENHANCED ACCUMULATION OF TREHALOSE IN PLANTS</b> (57) Abstract  The invention provides a process for producing trehalose in plant cells capable of producing trehalase by growing plant cells having the genetic information required for the production of trehalose and trehalase, or cultivating a plant or a part thereof comprising such plant cells, characterised in that said plant cells are grown, or said plant or a part thereof is cultivated in the presence of a trehalase inhibitor.</p>		

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## ENHANCED ACCUMULATION OF TREHALOSE IN PLANTS

## FIELD OF THE INVENTION

The invention relates to a method for the production of trehalose  
5 in plant cells, and plants. The invention is particularly related to a  
method for increasing the levels of trehalose accumulation in plants  
capable of producing trehalose. The invention further comprises higher  
plants, preferably *Angiospermae*, and parts thereof, which as a result of  
such methods, contain relatively high levels of trehalose. The invention  
10 further relates to plant cells, plants or parts thereof according to the  
invention obtained after processing thereof.

## STATE OF THE ART

Trehalose is a general name given to D-glucosyl D-glucosides which  
15 comprise disaccharides based on two  $\alpha$ -,  $\alpha$ , $\beta$ - and  $\beta$ , $\beta$ -linked glucose  
molecules. Trehalose, and especially  $\alpha$ -trehalose  
1-(O- $\alpha$ -D-glucopyranosyl)-1'-O- $\alpha$ -D-glucopyranose) is a widespread  
naturally occurring disaccharide. However, trehalose is not generally  
found in plants, apart from a few exceptions, such as the plant species  
20 *Selaginella lepidophylla* (*Lycophyta*) and *Myrothamnus flabellifolia*. Apart  
from these species, trehalose is found in root nodules of the *Leguminosae*  
(*Spermatophytae*, *Angiospermae*), wherein it is synthesized by bacteroids;  
the trehalose so produced is capable of diffusing into the root cells.  
Apart from these accidental occurrences, plant species belonging to the  
25 *Spermatophyta* apparently lack the ability to produce and/or accumulate  
trehalose.

In International patent application WO 95/01446, filed on June 30,  
1994 in the name of MOGEN International NV, a method is described for  
providing plants not naturally capable of producing trehalose with the  
30 capacity to do so. The method comprises introducing into the cells of  
said plants a recombinant polynucleotide encoding trehalose phosphate  
synthase under the control of regulatory elements necessary for  
expression of said recombinant DNA in plant cells. In one embodiment,  
tobacco and potato plants had been transformed with a recombinant  
35 polynucleotide encoding TPS from *E. coli*, under the control of the CaMV  
35S RNA promoter. Levels of trehalose accumulation in these plants tended

to be rather low.

In spite of the absence of trehalose as a substrate in most higher plant species, the occurrence of trehalose-degrading activity has been reported for a considerable number of higher plant species, including  
5 those known to lack trehalose. The responsible activity could be attributed to a trehalase enzyme.

Reports suggest that trehalose, when fed to plant shoots grown in vitro is toxic or inhibitory to the growth of plant cells (Veluthambi K. et al., 1981, Plant Physiol. 68, 1369-1374). Plant cells producing low  
10 trehalase levels were found to be generally more sensitive to the adverse effects of trehalose, than plants exhibiting a higher level of trehalase activity. Trehalose-analogs, such as trehalose-amines were used to inhibit trehalase activity in shoots, making it possible to study the effects of trehalose fed to plant cells. Plant shoots which produce  
15 relatively high amounts of trehalase were adversely affected by the addition of trehalase inhibitors. Inhibition of trehalase activity in homogenates of callus and suspension culture of various *Angiospermae* using Validamycin is disclosed by Kendall et al., 1990, Phytochemistry 29, 2525-2582.

20 It is an object of the present invention to provide plants and plant parts capable of producing and accumulating trehalose, while keeping any adverse effects that may arise from the accumulation of trehalose within acceptable limits.

## 25 SUMMARY OF THE INVENTION

The invention provides a process for producing trehalose in plant cells capable of producing trehalase by growing plant cells having the genetic information required for the production of trehalose and trehalase, or cultivating a plant or a part thereof comprising such plant  
30 cells, characterised in that said plant cells are grown, or said plant or a part thereof, is cultivated in the presence of a trehalase inhibitor. Preferred plants or plant parts or plant cells have been genetically altered so as to contain a chimeric trehalose phosphate synthase gene in a plant expressible form. According to one embodiment said trehalose  
35 phosphate synthase gene comprises an open reading frame encoding trehalose phosphate synthase from *E. coli* in plant expressible form.

According to a further aspect of the invention, plants have been genetically altered so as to produce trehalose preferentially in certain tissues or parts, such as (micro-)tubers of potato. According to one embodiment the open reading frame encoding trehalose phosphate synthase  
5 from *E. coli* is downstream of the potato patatin promoter, to provide for preferential expression of the gene in tubers and micro-tubers of *Solanum tuberosum*.

According to another aspect of the invention the plants are cultivated *in vitro*, for example in hydroculture.

10 According to another preferred embodiment said trehalase inhibitor comprises validamycin A in a form suitable for uptake by said plant cells, preferably in a concentration between 100 nM and 10 mM, preferably between 0.1 and 1 mM, in aqueous solution.

Equally suitable said trehalase inhibition can be formed by  
15 transformation of said plant with the antisense gene to the gene encoding the information for trehalase.

Also suitable as trehalase inhibitor is the 86 kD protein from the american cockroach (*Periplaneta americana*). This protein can be administered to a plant in a form suitable for uptake, and also it is  
20 possible that the plants are transformed with DNA coding for said protein.

The invention further provides plants and plant parts which accumulate trehalose in an amount above 0.01 % (fresh weight), preferably of a *Solanaceae* species, in particular *Solanum tuberosum* or  
25 *Nicotiana tabacum*, in particular a micro-tuber of *Solanum tuberosum* containing trehalose.

The invention also comprises the use of a plant, or plant part, according to the invention for extracting trehalose, as well as the use thereof in a process of forced extraction of water from said plant or  
30 plant part. According to yet another embodiment of the invention a chimaeric plant expressible gene is provided, comprising in sequence a transcription initiation region obtainable from a gene, preferentially expressed in a plant part, particularly the patatin gene from *Solanum tuberosum*, a 5'-untranslated leader, an open reading frame encoding a  
35 trehalose phosphate synthase activity, and downstream of said open reading frame a transcriptional terminator region.

According to yet another embodiment of the invention a chimaeric plant expressible gene is provided, comprising in sequence a transcription initiation region obtainable from a gene, preferentially expressed in a plant part, particularly the patatin gene from *Solanum tuberosum*, a 5'-untranslated leader, an open reading frame encoding a trehalase coupled in the antisense orientation, and downstream of said open reading frame a transcriptional terminator region. A preferred plant expressible gene according to the invention is one wherein said transcriptional terminator region is obtainable from the proteinase inhibitor-II gene of *Solanum tuberosum*. The invention also provided vectors and recombinant plant genomes comprising a chimaeric plant expressible gene according to the invention, as well as a plant cell having a recombinant genome, a plant or a part thereof, consisting essentially of cells. A further preferred plant species according to this aspect is *Solanum tuberosum*, and a micro-tuber thereof.

The invention further provides a process for obtaining trehalose, comprising the steps of growing plant cells according to the invention or cultivating a plant according to the invention and extracting trehalose from said plant cells, plants or parts.

The following figures further illustrate the invention.

#### DESCRIPTION OF THE FIGURES

Figure 1. Schematic representation of binary vector pMOG799.

Figure 2. Schematic representation of binary vector pMOG845.

Figure 3. Schematic representation of parts of the sucrose and starch biosynthetic pathways in plant sink tissues. The figure shows that carbohydrate produced in the leaf by photosynthesis is transported via the phloem tissue in the form of sucrose. Upon entering the sink it is unloaded by a membrane bound invertase activity to yield the monosugars glucose and fructose. By the action of a number of enzymatic steps these monosugars are converted to starch and/or sucrose as roughly shown here. The glucose metabolites G6P and UDPG are believed to be used as the substrates for the TPS-enzyme engineered into the plant by introduction of the plant expressible otsA gene. The figure shows how the amount of UDPG and G6P available as substrate is increased by reducing the levels of the enzymes SPS and AGPase. Their inhibition is marked with a cross.



Figure 4. Alignments for maximal amino acid similarities of neutral trehalase from *S. cerevisiae* with periplasmatic trehalase from *E. coli*, small intestinal trehalase from rabbit and trehalase from pupal midgut of the silkworm, *Bombyx mori*. Identical residues among all trehalase enzymes are indicated in ***bold italics*** typeface. Conserved regions of the amino acid sequences were aligned to give the best fit. Gap's in the amino acid sequence are represented by dashes.

Positions of degenerated primers based on conserved amino acids are indicated by dashed arrows.

#### DETAILED DESCRIPTION OF THE INVENTION

According to the present invention it has been found that the accumulation of an increased level of trehalose in plants and plant parts is feasible, without causing too drastic effects on the viability of the plant or plant parts. This important finding can be exploited by adapting plant systems to produce and/or accumulate high levels of trehalose at lower cost.

According to one embodiment of the invention the accumulation of increased levels of trehalose is achieved by inhibiting endogenous trehalases. Inhibition of trehalases can be performed basically in two ways: by administration of trehalase inhibitors exogenously, and by the production of trehalase inhibitors endogenously, for instance by transforming the plants with DNA sequences coding for trehalase inhibitors.

This inhibition can be equally well applied to plants which are transformed with enzymes which enable the production of trehalose, but also to plants which are able to synthesize trehalose naturally.

According to this first embodiment of the invention, trehalase inhibitors are administered to the plant system exogenously. Examples of trehalase inhibitors that may be used in such a process according to the invention are trehazolin produced in *Micromonospora*, strain SANK 62390 (Ando et al., 1991, J. Antibiot. 44, 1165-1168), validoxylamine A, B, G, D-glucosyl-Dihydrovalidoxylamine A, L-ido-Dihydrovalidoxylamin A, Deoxynojirimycin (Kameda et al., 1987, J. Antibiot. 40(4), 563-565), 5-epi-trehazolin (Trehalostatin) (Kobayashi Y. et al., 1994, J. Antibiot. 47, 932-938), castanospermin (Salleh H.M. & Honek J.F. March 1990, FEBS 262(2), 359-362) and the 86kD protein from the american cockroach (*Periplaneta*

americana) (Hayakawa et al., 1989, J. Biol. Chem. 264(27), 16165-16169).

A preferred trehalase inhibitor according to the invention is validamycin A (1,5,6-trideoxy-3-o- $\beta$ -D-glucopyranosyl-5-(hydroxymethyl)-1-[[4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl]amino]-D-chiro-inositol).

5 Trehalase inhibitors are administered to plants or plant parts, or plant cell cultures, in a form suitable for uptake by the plants, plant parts or cultures. Typically the trehalase inhibitor is in the form of an aqueous solution of between 100 nM and 10 mM of active ingredient, preferably between 0.1 and 1 mM. Aqueous solutions may be applied to plants or plant  
10 parts by spraying on leaves, watering, adding it to the medium of a hydroculture, and the like. Another suitable formulation of validamycin is solacol, a commercially available agricultural formulation (Takeda Chem. Indust., Tokyo).

Alternatively, or in addition to using exogenously administered  
15 trehalase inhibitors, trehalase inhibitors may be provided by introducing the genetic information coding therefor. One form of such in-built trehalase inhibitor may consist of a genetic construct causing the production of RNA that is sufficiently complementary to endogenous RNA encoding for trehalase to interact with said endogenous transcript, thereby  
20 inhibiting the expression of said transcript. This so-called "antisense approach" is well known in the art (vide inter alia EP 0 240 208 A and the Examples to inhibit SPS disclosed in WO 95/01446).

A gene coding for trehalase has been isolated from a potato cDNA library and sequenced. The predicted amino acid sequence of trehalase as  
25 shown in SEQIDNO:10 is derived from the nucleotide sequence depicted in SEQIDNO: 9. As is well known in the biological arts amino acid sequences of equivalent enzymes can differ between species. It is emphasized that the difference between the known trehalase sequences and plant trehalase sequence makes it very questionable if such trehalase sequence used in an  
30 antisense approach is capable of inhibiting trehalase expression in planta.

Of course the most preferred embodiment of the invention is obtained by transforming a plant with the antisense trehalase gene which matches exactly with the endogenous trehalase gene. However, sequences which have a high degree of homology can also be used. Thus, the antisense  
35 trehalase gene to be used for the transformation of potato will be directed against the nucleotide sequence depicted in SEQIDNO: 9.

It is usually enough to express only part of the homologous gene in the antisense orientation, in order to achieve effective inhibition of expression of the endogenous trehalase (vide Van der Krol et al., 1990, Plant Molecular Biology, 14, 457-466).

5           Trehalase gene sequences of other plants can be elucidated in two different ways. One of the strategies is to use the isolated potato cDNA clone as a probe to screen a cDNA library containing the cDNA of the desired plant species. Positive reacting clones can then be isolated and subcloned into suitable vectors.

10           A second strategy to identify such genes is by purifying the proteins which are involved in trehalose degradation. An example for such a strategy is the purification of a protein with acid invertase activity from potato (*Solanum tuberosum* L.) tubers (Burch et al., Phytochemistry, Vol. 31, No.6, pp. 1901-1904, 1992). The obtained protein  
15           preparation also exhibits trehalose hydrolysing activity. Disaccharide hydrolysing activity of protein preparations obtained after purification steps can be monitored as described by Dahlqvist (Analytical Biochemistry 7, 18-25, 1964).

          After purifying the protein(s) with trehalose hydrolysing  
20           activity to homogeneity, the N-terminal amino acid sequence or the sequence of internal fragments after protein digestion is determined. These sequences enable the design of oligonucleotide probes which are used in a polymerase chain reaction (PCR) or hybridization experiments to isolate the corresponding mRNAs using standard molecular cloning techniques.

25           An isolated cDNA encoding a trehalose degrading enzyme is subsequently fused to a promoter sequence in such a way that transcription results in the synthesis of antisense mRNA.

          Another form of such an in-built trehalase inhibitor may consist of a genetic construct causing the production of a protein that is able to  
30           inhibit trehalase activity in plants. A proteinaceous inhibitor of trehalase has been isolated and purified from the serum of resting adult american cockroaches (*Periplaneta americana*) (Hayakawa et al., supra). This protein, of which the sequence partly has been described in said publication, can be made expressable by isolation of the gene coding for  
35           the protein, fusion of the gene to a suitable promoter, and transformation of said fused gene into the plant according to standard molecular

biological methods.

A promoter may be selected from any gene capable of driving transcription in plant cells.

If trehalose accumulation is only desired in certain plant parts, such as potato (mini-)tubers, the trehalase inhibitory DNA construct (e.g. the antisense construct) comprises a promoter fragment that is preferentially expressed in (mini-)tubers, allowing endogenous trehalase levels in the remainder of the plant's cells to be substantially unaffected. Thus, any negative effects of trehalose to neighbouring plant cells due to trehalose diffusion, is counteracted by unaffected endogenous trehalase activity in the remainder of the plant.

In the Example illustrating the invention, wherein trehalose phosphate synthase is produced under the control of the patatin promoter fragment, also the trehalase-inhibitory construct may comprise a promoter fragment of the patatin-gene.

*Mutatis mutandis* if trehalose is to be accumulated in tomato fruit, both a plant expressible trehalose phosphate synthase gene, which is at least expressed in the tomato fruit is to be used, as well as a plant expressible trehalase-inhibitory DNA construct, which should be expressed preferentially in the fruit, and preferably not, or not substantially, outside the fruit. An example of a promoter fragment that may be used to drive expression of DNA-constructs preferentially in tomato fruit is disclosed in EP 0 409 629 A1. Numerous modifications of this aspect of the invention, that do not depart from the scope of this invention, are readily envisaged by persons having ordinary skill in the art to which this invention pertains.

An alternative method to block the synthesis of undesired enzymatic activity such as caused by endogenous trehalase is the introduction into the genome of the plant host of an additional copy of said endogenous trehalase gene. It is often observed that the presence of a transgene copy of an endogenous gene silences the expression of both the endogenous gene and the transgene (EP 0 465 572 A1).

According to one embodiment of the invention accumulation of trehalose is brought about in plants wherein the capacity of producing trehalose has been introduced by introduction of a plant expressible gene construct encoding trehalose phosphate synthase (TPS).

Any trehalose phosphate synthase gene under the control of regulatory elements necessary for expression of DNA in plant cells, either specifically or constitutively, may be used, as long as it is capable of producing an active trehalose phosphate synthase activity. A preferred open  
5 reading frame according to the invention is one encoding a TPS-enzyme as represented in SEQIDNO: 2. It is well known that more than one DNA sequence may encode an identical enzyme, which fact is caused by the degeneracy of the genetic code. If desired, the open reading frame encoding the trehalose phosphate synthase activity may be adapted to codon usage in the host plant  
10 of choice, but this is not a requirement.

The isolated nucleic acid sequence represented by SEQIDNO: 2, may be used to identify trehalose phosphate synthase activities in other organisms and subsequently isolating and cloning them, by hybridizing DNA from other sources with a DNA- or RNA fragment obtainable from the *E. coli*  
15 gene. Preferably, such DNA sequences are screened by hybridizing under more or less stringent conditions (such as temperature and ionic strength of the hybridization mixture). Whether or not conditions are stringent also depends on the nature of the hybridization, i.e. DNA:DNA, DNA:RNA, RNA:RNA, as well as the length of the shortest hybridizing fragment. Those of skill  
20 in the art are readily capable of establishing a hybridization regime stringent enough to isolate TPS genes, while avoiding aspecific hybridization. As genes involved in trehalose synthesis from other sources become available these can be used in a similar way to obtain a plant expressible trehalose phosphate synthase gene according to the invention.

25 Sources for isolating trehalose phosphate synthase activities include microorganisms (e.g. bacteria, yeast, fungi), plants, animals, and the like. Isolated DNA sequences encoding trehalose phosphate activity from other sources may be used likewise in a method for producing trehalose according to the invention. As an example, genes for producing trehalose  
30 from yeast are disclosed in WO 93/17093.

The invention also encompasses nucleic acid sequences which have been obtained by modifying the nucleic acid sequence represented in SEQIDNO: 2 by mutating one or more codons so that it results in amino acid changes in the encoded protein, as long as mutation of the amino acid  
35 sequence does not entirely abolish trehalose phosphate synthase activity.

According to another embodiment of the invention, plants are

genetically altered to produce and accumulate trehalose in specific parts of the plant, which were selected on the basis of considerations such as substrate availability for the TPS-enzyme, insensitivity of the plant part to any putative adverse effects of trehalose on plant cell functioning, and the like. A preferred site of TPS- enzyme expression are starch storage parts of plants. In particular microtubers potato are considered to be suitable plant parts. A preferred promoter to achieve selective TPS-enzyme expression in microtubers and tubers of potato is obtainable from the region upstream of the open reading frame of the patatin gene of potato (*Solanum tuberosum*).

Plants may be further modified by introducing additional genes that encode phosphatases that are capable of the conversion of trehalose phosphate into trehalose. At least in potato tubers or micro-tubers, potato leaves and tobacco leaves and roots, endogenous phosphatase activity appears to be present, so that the introduction of a trehalose phosphate phosphatase (TPP) gene is not an absolute requirement.

According to another embodiment of the invention, trehalose accumulation is further enhanced by the inhibition of endogenous genes in order to enhance substrate availability for the trehalose phosphate synthase, as exemplified herein with the inhibition of endogenous sucrose phosphate synthase gene and the ADP-Glucose pyrophosphorylase gene (AGP-ase). Inhibition of undesired endogenous enzyme activity is achieved in a number of ways, the choice of which is not critical to the invention. Preferably gene inhibition is achieved through the so-called 'antisense approach'. Herein a DNA sequence is expressed which produces an RNA that is at least partially complementary to the RNA which encodes the enzymatic activity that is to be blocked (e.g. AGP-ase or SPS (Sucrose Phosphate Synthase), in the examples). It is preferred to use homologous antisense genes as these are more efficient than heterologous genes. The isolation of an antisense SPS gene from potato using a maize SPS-gene sequence as probe serves to illustrate the feasibility of this strategy. It is not meant to indicate that, for practicing the invention the use of homologous antisense fragments is required. An alternative method to block the synthesis of undesired enzymatic activities is the introduction into the genome of the plant host of an additional copy of an endogenous gene present in the plant host. It is often observed that such an additional copy of a gene silences

the endogenous gene: this effect is referred to in the literature as the co-suppressive effect, or co-suppression. Details of the procedure of enhancing substrate availability are provided in the Examples of WO 95/01446, incorporated by reference herein.

5 Preferred plant hosts among the spermatophyta are the Angiospermae, notably the Dicotyledoneae, comprising inter alia the Solanaceae as a representative family, and the Monocotyledoneae, comprising inter alia the Gramineae as a representative family. Suitable host plants, as defined in the context of the present invention include plants (as well  
10 as parts and cells of said plants) and their progeny which have been genetically modified using recombinant DNA techniques to cause or enhance production of trehalose in the desired plant or plant organ; these plants may be used directly (e.g. the plant species which produce edible parts) in processing or the trehalose may be extracted and/or purified from said  
15 host. Crops with edible parts according to the invention include those which have flowers such as cauliflower (*Brassica oleracea*), artichoke (*Cynara scolymus*), fruits such as apple (*Malus*, e.g. *domesticus*), banana (*Musa*, e.g. *acuminata*), berries (such as the currant, *Ribes*, e.g. *rubrum*), cherries (such as the sweet cherry, *Prunus*, e.g. *avium*), cucumber (*Cucumis*,  
20 e.g. *sativus*), grape (*Vitis*, e.g. *vinifera*), lemon (*Citrus limon*), melon (*Cucumis melo*), nuts (such as the walnut, *Juglans*, e.g. *regia*; peanut, *Arachis hypogaeae*), orange (*Citrus*, e.g. *maxima*), peach (*Prunus*, e.g. *persica*), pear (*Pyra*, e.g. *communis*), pepper (*Solanum*, e.g. *capsicum*), plum (*Prunus*, e.g. *domestica*), strawberry (*Fragaria*, e.g. *moschata*), tomato  
25 (*Lycopersicon*, e.g. *esculentum*), leafs, such as alfalfa (*Medicago sativa*), cabbages (such as *Brassica oleracea*), endive (*Cichoreum*, e.g. *endivia*), leek (*Allium porrum*), lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), tobacco (*Nicotiana tabacum*), roots, such as arrowroot (*Maranta arundinacea*), beet (*Beta vulgaris*), carrot (*Daucus carota*),  
30 cassava (*Manihot esculenta*), turnip (*Brassica rapa*), radish (*Raphanus sativus*), yam (*Dioscorea esculenta*), sweet potato (*Ipomoea batatas*) and seeds, such as bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), soybean (*Glycin max*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), corn (*Zea mays*), rice (*Oryza sativa*), tubers, such as kohlrabi (*Brassica oleracea*), potato (*Solanum tuberosum*), and the like. The edible parts may  
35 be conserved by drying in the presence of enhanced trehalose levels

produced therein due to the presence of a plant expressible trehalose phosphate synthase gene.

The method of introducing the plant expressible trehalose-phosphate synthase gene, or any other sense or antisense gene into a recipient plant cell is not crucial, as long as the gene is expressed in said plant cell. The use of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* - mediated transformation is preferred, but other procedures are available for the introduction of DNA into plant cells. Examples are transformation of protoplasts using the calcium/polyethylene glycol method, electroporation microinjection and DNA-coated particle bombardment (Potrykus, 1990, Bio/Technol. 8, 535-542). Also combinations of *Agrobacterium* and coated particle bombardment may be used. Also transformation protocols involving other living vectors than *Agrobacterium* may be used, such as viral vectors (e.g. from the Cauliflower Mosaic Virus (CaMV) and or combinations of *Agrobacterium* and viral vectors, a procedure referred to as agroinfection (Grimsley N. et al., 8 January 1987, Nature 325, 177-179). After selection and/or screening, the protoplasts, cells or plant parts that have been transformed are regenerated into whole plants, using methods known in the art (Horsch et al., 1985, Science 225, 1229-1231).

The development of reproducible tissue culture systems for monocotyledonous crops, together with methods for introduction of genetic material into plant cells has facilitated transformation. Presently, preferred methods for transformation of monocot species are microprojectile bombardment of explants or suspension cells, and direct DNA uptake or electroporation (Shimamoto, et al., 1989, Nature 338, 274-276). Transgenic maize plants have been obtained by introducing the *Streptomyces hygroscopicus* bar-gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microprojectile bombardment (Gordon-Kamm, 1990, Plant Cell, 2, 603-618). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee, 1989, Plant Mol. Biol. 13, 21-30). Wheat plants have been regenerated from embryogenic suspension culture by selecting only the aged compact and nodular embryogenic callus tissues for the establishment of the embryogenic suspension cultures



(Vasil, 1990 Bio/Technol. 8, 429-434).

Monocotyledonous plants, including commercially important crops such as corn and rice may be obtained by *Agrobacterium*-mediated transformation according to Gould J, Michael D, Hasegawa O, Ulian EC, Peterson G, Smith RH, (1991) Plant. Physiol. 95, 426-434; Hiei Y. et al., The Plant Journal 6(2), 271-282 and European patent 159 418 B1.

Suitable DNA sequences for control of expression of the plant expressible genes (including marker genes), such as transcriptional initiation regions, enhancers, non-transcribed leaders and the like, may be derived from any gene that is expressed in a plant cell. Also intended are hybrid promoters combining functional portions of various promoters, or synthetic equivalents thereof. Apart from constitutive promoters, inducible promoters, or promoters otherwise regulated in their expression pattern, e.g. developmentally or cell-type specific, may be used to control expression of the plant expressible genes according to the invention as long as they are expressed in plant parts that contain substrate for TPS.

To select or screen for transformed cells, it is preferred to include a marker gene linked to the plant expressible gene according to the invention to be transferred to a plant cell. The choice of a suitable marker gene in plant transformation is well within the scope of the average skilled worker; some examples of routinely used marker genes are the neomycin phosphotransferase genes conferring resistance to kanamycin (EP-B 131 623), the Glutathion-S-transferase gene from rat liver conferring resistance to glutathione derived herbicides (EP-A 256 223), glutamine synthetase conferring upon overexpression resistance to glutamine synthetase inhibitors such as phosphinothricin (WO87/05327), the acetyl transferase gene from *Streptomyces viridochromogenes* conferring resistance to the selective agent phosphinothricin (EP-A 275 957), the gene encoding a 5-enolshikimate-3- phosphate synthase (EPSPS) conferring tolerance to N-phosphonomethylglycine, the bar gene conferring resistance against Bialaphos (e.g. WO 91/02071) and the like. The actual choice of the marker is not crucial as long as it is functional (i.e. selective) in combination with the plant cells of choice.

The marker gene and the gene of interest do not have to be linked, since co-transformation of unlinked genes (U.S. Patent 4,399,216) is also an efficient process in plant transformation.

Preferred plant material for transformation, especially for dicotyledonous crops are leaf-discs which can be readily transformed and have good regenerative capability (Horsch R.B. et al., (1985) Science 227, 1229-1231).

- 5                   It is immaterial to the invention how the presence of two or more genes in the same plant is effected. This can *inter alia* done be achieved by one of the following methods:
- (a) transformation of the plant line with a multigene construct containing more than one gene to be introduced,
  - 10 (b) co-transforming different constructs to the same plant line simultaneously,
  - (c) subsequent rounds of transformation of the same plant with the genes to be introduced,
  - (d) crossing two plants each of which contains a different gene to be
  - 15 introduced into the same plant, or
  - (e) combinations thereof.

                  The field of application of the invention lies both in agriculture and horticulture, for instance due to improved properties of the modified plants as such (e.g. stress tolerance, such as cold tolerance,

20 and preferably drought resistance, and increase in post-harvest quality and shelf-life of plants and plant products), as well as in any form of industry where trehalose is or will be applied in a process of forced water extraction, such as drying or freeze drying. Trehalose can be used or sold as such, for instance in purified form or in admixtures, or in the form of

25 a plant product, such as tuber, a fruit, a flower containing the trehalose, either in native state or in (partially) dehydrated form, and the like. Plant parts harbouring (increased levels of) trehalose phosphate or trehalose may be used or sold as such or processed without the need to add trehalose.

- 30                   Also trehalose can be extracted and/or purified from the plants or plant parts producing it and subsequently used in an industrial process. In the food industries trehalose can be employed by adding trehalose to foods before drying. Drying of foods is an important method of preservation. Trehalose seems especially useful to conserve food products
- 35 through conventional air-drying, and to allow for fast reconstitution upon addition of water of a high quality product (Roser et al., July 1991,

Trends in Food Science and Technology, pp. 166-169). The benefits include retention of natural flavors/fragrances, taste of fresh product, and nutritional value (proteins and vitamins). It has been shown that trehalose has the ability to stabilize proteins e.g. vaccines, enzymes and membranes, and to form a chemically inert, stable glass. The low water activity of such thoroughly dried food products prevents chemical reactions, that could cause spoilage.

Field crops like corn, cassava, potato, sugar beet and sugarcane have since long been used as a natural source for bulk carbohydrate production (starches and sucrose). The production of trehalose in such crops, facilitated by genetic engineering of the trehalose-biosynthetic pathway into these plant species, would allow the exploitation of such engineered crops for trehalose production.

Trehalose is also used in drying or storage of biological macromolecules, such as peptides, enzymes, polynucleotides and the like.

All references cited in this specification are indicative of the level of skill in the art to which the invention pertains. All publications, whether patents or otherwise, referred to previously or later in this specification are herein incorporated by reference as if each of them was individually incorporated by reference. In particular WO 95/01446, cited herein, describing the production of trehalose in higher plants by genetic manipulation is herein incorporated by reference.

The Examples given below illustrate the invention and are in no way intended to indicate the limits of the scope of the invention.

## **Experimental**

### **DNA manipulations**

All DNA procedures (DNA isolation from *E.coli*, restriction, ligation, transformation, etc.) are performed according to standard protocols (Sambrook et al. (1989) Molecular Cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, CSH, New York).

### **Strains**

In all examples *E.coli* K-12 strain DH5 $\alpha$  is used for cloning. The *Agrobacterium tumefaciens* strains used for plant transformation experiments

are EHA 105 and MOG 101 (Hood et al. 1993, Trans. Research 2, 208-218)

Construction of Agrobacterium strain MOG101

A binary vector system (Hoekema A., Hirsch, P.R., Hooykaas, P.J.J., and Schilperoort, R.A. (1983) Nature 303, 179) is used to transfer gene constructs into potato and tobacco plants. The helper plasmid conferring the *Agrobacterium tumefaciens* virulence functions is derived from the octopine Ti-plasmid pTiB6. MOG101 is an *Agrobacterium tumefaciens* strain carrying a non-oncogenic Ti-plasmid (Koekman et al. 1982, supra) from which the entire T-region is deleted and substituted by a bacterial Spectinomycin resistance marker from transposon Tn1831 (Hooykaas et al., 1980 Plasmid 4, 64-75). The Ti-plasmid pTiB6 contains two adjacent T-regions, TL (T-left) and TR (T-right). To obtain a derivative lacking the TL- and TR-regions, we constructed intermediate vector pMOG579. Plasmid pMOG579 is a pBR322 derivative which contains 2 Ti-plasmid fragments homologous to the fragments located left and right outside the T-regions of pTiB6. The 2 fragments are separated in pMOG579 by a 2.5 kb BamHI - HindIII fragment from transposon Tn1831 (Hooykaas et al., 1980 Plasmid 4, 64-75) carrying the spectinomycin resistance marker. The plasmid is introduced into *Agrobacterium tumefaciens* strain LBA1010 [C58-C9 (pTiB6) = a cured C58 strain in which pTiB6 is introduced (Koekman et al. (1982), supra), by triparental mating from *E.coli*, using HB101 8pRK2013 as a helper. Transconjugants are selected for resistance to Rifampicin (20 mg/l) and spectinomycin (250 mg/l). A double recombination between pMOG579 and pTiB6 resulted in loss of carbenicillin resistance (the pBR322 marker) and deletion of the entire T-region. Of 5000 spectinomycin resistant transconjugants replica plated onto carbenicillin (100 mg/l) 2 are found sensitive. Southern analysis (not shown) showed that a double crossing over event had deleted the entire T-region. The resulting strain is called MOG101. This strain and its construction is analogous to strain GV2260 (Deblaere et al. 1985, Nucl. Acid Res. 13, 4777-4788).

An alternative helper strain for MOG101 is e.g. LBA4404; this strain can also suitably be used for introduction of a binary plasmid, such as pMOG799 and subsequent plant transformation. Other suitable helper strains are readily available.

Isolation of a patatin promoter/construction of pMOG546

A patatin promoter fragment is isolated from chromosomal DNA of *Solanum tuberosum* cv. Bintje using the polymerase chain reaction. A set of oligonucleotides, complementary to the sequence of the upstream region of the  $\lambda$ pat21 patatin gene (Bevan, M., Barker, R., Goldsbrough, A., Jarvis, M., Kavanagh, T. and Iturriaga, G. (1986) *Nucleic Acids Res.* **14**: 5564-5566), is synthesized consisting of the following sequences:

10                   5' AAG CTT ATG TTG CCA TAT AGA GTA G 3'      PatB33.2 (SEQIDNO:3)  
                  5' GTA GTT GCC ATG GTG CAA ATG TTC 3'      PatATG.2 (SEQIDNO:4)

These primers are used to PCR amplify a DNA fragment of 1123bp, using chromosomal DNA isolated from potato cv. Bintje as a template. The amplified fragment shows a high degree of similarity to the  $\lambda$ pat21 patatin sequence and is cloned using EcoRI linkers into a pUC18 vector resulting in plasmid pMOG546.

Construction of pMOG 799

pMOG 799 harbours the TPS gene from *E. coli* under control of the double enhanced 35S Cauliflower Mosaic promoter. The construction of this binary vector is described in detail in International patent application PCT/EP94/02167, incorporated herein by reference. A sample of an *E. coli* strain harbouring pMOG799 has been deposited under the Budapest Treaty at the Centraal Bureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, The Netherlands, on Monday 23 August, 1993: the Accession Number given by the International Depositary Institution is CBS 430.93.

Construction of pMOG845.

Plasmid pMOG546 containing the patatin promoter is digested with NcoI-KpnI, incubated with *E. coli* DNA polymerase I in the presence of dATP and dCTP thereby destroying the NcoI and KpnI site and subsequently relegated. From the resulting vector a 1.1kb EcoRI-SmaI fragment containing the patatin promoter is isolated and cloned into pMOG798 (described in detail in PCT/EP94/02167) linearized with SmaI-EcoRI consequently exchanging the 35S CaMV promoter for the patatin promoter. The resulting vector is linearized with HindIII and ligated with the following oligonucleotide duplex:

	(HindIII)	PstI	KpnI	HindIII	
	5'	AGCT CTGCAG TGA GGTACC A	3'	TCV 11 (SEQIDNO:5)	
5	3'	GACGTC ACT CCATGG TTCGA	5'	TCV 12 (SEQIDNO:6)	

After checking the orientation of the introduced oligonucleotide duplex, the resulting vector is linearized with PstI-HindIII followed by the insertion of a 950bp PstI-HindIII fragment harbouring the potato proteinase inhibitor II terminator (PotPiII) (An, G., Mitra, A., Choi, H.K., Costa, M.A., An, K., Thornburg, R. W. and Ryan, C.A. (1989) *The Plant Cell* 1: 115-122 ). The PotPiII terminator is isolated by PCR amplification using chromosomal DNA isolated from potato cv. Desiree as a template and the following set of oligonucleotides:

15	5'	GTACCCTGCAGTGTGACCCTAGAC	3'	TCV 15 (SEQIDNO:7)
	5'	TCGATTCATAGAAGCTTAGAT	3'	TCV 16 (SEQIDNO:8)

The TPS expression cassette is subsequently cloned as a EcoRI-HindIII fragment into the binary vector pMOG402 resulting in pMOG845 (fig. 4). A sample of *E.coli* Dh $\alpha$  strain, harbouring pMOG845 has been deposited at the Centraal Bureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, The Netherlands, on January 4, 1995; the Accession Number given by the International Depositary Institution is CBS 101.95.

25.

#### Triparental matings

The binary vectors are mobilized in triparental matings with the *E. coli* strain HB101 containing plasmid pRK2013 (Ditta G., Stanfield, S., Corbin, D., and Helinski, D.R. et al. (1980) *Proc. Natl. Acad. Sci. USA* 77, 7347) into *Agrobacterium tumefaciens* strain MOG101 or EHA105 and used for transformation.

#### Transformation of tobacco (*Nicotiana tabacum* SR1)

Tobacco is transformed by cocultivation of plant tissue with *Agrobacterium tumefaciens* strain MOG101 containing the binary vector of interest as described. Transformation is carried out using cocultivation of tobacco

(*Nicotiana tabacum* SR1) leaf disks as described by Horsch et al. 1985, Science 227, 1229-1231. Transgenic plants are regenerated from shoots that grow on selection medium containing kanamycin, rooted and transferred to soil.

5

#### Transformation of potato

Potato (*Solanum tuberosum* cv. Kardal) is transformed with the *Agrobacterium* strain EHA 105 containing the binary vector of interest. The basic culture medium is MS30R3 medium consisting of MS salts (Murashige, T. and Skoog, F. 10 (1962) Physiol. Plan. 14, 473), R3 vitamins (Ooms et al. (1987) Theor. Appl. Genet. 73, 744), 30 g/l sucrose, 0.5 g/l MES with final pH 5.8 (adjusted with KOH) solidified when necessary with 8 g/l Daichin agar. Tubers of *Solanum tuberosum* cv. Kardal are peeled and surface sterilized by burning them in 96% ethanol for 5 seconds. Extinguish the flames in sterile 15 water and cut slices of approximately 2 mm thickness. Disks are cut with a bore from the vascular tissue and incubated for 20 minutes in MS30R3 medium containing 1-5 x10<sup>8</sup> bacteria/ml of *Agrobacterium* EHA 105 containing the binary vector. Wash the tuber discs with MS30R3 medium and transfer them to solidified postculture medium (PM). PM consists of M30R3 medium 20 supplemented with 3.5 mg/l zeatin riboside and 0.03 mg/l indole acetic acid (IAA). After two days, discs were transferred to fresh PM medium with 200 mg/l cefotaxim and 100 mg/l vancomycin. Three days later, the tuber discs are transferred to shoot induction medium (SIM) which consists of PM medium with 250 mg/l carbenicillin and 100 mg/l kanamycin. After 4-8 weeks, shoots 25 emerging from the discs are excised and placed on rooting medium (MS30R3-medium with 100 mg/l cefotaxim, 50 mg/l vancomycin and 50 mg/l kanamycin). The shoots are propagated axenically by meristem cuttings.

#### Induction of micro-tubers

30 Stem segments of *in vitro* potato plants harbouring an auxiliary meristem are transferred to micro-tuber inducing medium. Micro-tuber inducing medium contains 1 X MS-salts supplemented with R3 vitamins, 0.5 g/l MES (final pH= 5.8, adjusted with KOH) and solidified with 8 g/l Daishin agar, 60 g/l sucrose and 2.5 mg/l kinetin. After 3 to 5 weeks of growth in the dark at 35 24°C, micro-tubers are formed.

#### Trehalose assay

Trehalose was determined quantitatively by anion exchange chromatography with pulsed amperometric detection. Extracts were prepared by adding 1 ml boiling water to 1 g frozen material which was subsequently heated for 15' at 100°C. Samples (25 µl) were analyzed on a Dionex DX-300 liquid chromatograph equipped with a 4 x 250 mm Dionex 35391 carbopac PA-1 column and a 4 x 50 mm Dionex 43096 carbopac PA-1 precolumn. Elution was with 100 mM NaOH at 1 ml/min. Sugars were detected with a pulsed amperometric detector (Dionex, PAD-2). Commercially available trehalose (Sigma) was used as a standard.

#### Isolation of Validamycin A

Validamycin A is isolated from Solacol, a commercial agricultural formulation (Takeda Chem. Indust., Tokyo) as described by Kendall et al. (1990) *Phytochemistry*, Vol. 29, No. 8, pp. 2525-2528. The procedure involves ion exchange chromatography (QAE-Sephadex A-25 (Pharmacia), bed vol. 10 ml, equilibration buffer 0.2 mM Na-Pi pH 7) from a 3% agricultural formulation of Solacol. Loading 1 ml of Solacol on the column and eluting with water in 7 fractions, practically all Validamycin is recovered in fraction 4.

Based on a 100% recovery, using this procedure, the concentration of Validamycin A was adjusted to  $110^{-3}$  M in MS-buffer, for use in trehalose accumulation tests.

Alternatively, Validamycin A and B may be purified directly from *Streptomyces hygroscopicus* var. *limoneus*, as described by Iwasa T. et al., 1971, in *The Journal of Antibiotics* 24(2), 119-123, the content of which is incorporated herein by reference.

#### Construction of pMOG1027

pMOG1027 harbours the trehalase gene from *Solanum tuberosum* cv. Kardal in the reversed orientation under control of the double enhanced 35S Cauliflower Mosaic promoter. The construction of this vector is very similar to the construction of pMOG799 and can be performed by any person skilled in the art. After mobilization of this binary vector by triparental mating to *Agrobacterium*, this strain can be used to transform plant cells and to generate transgenic plants having reduced levels of trehalase



activity.

#### Construction of pMOG1028

pMOG1028 harbours the trehalase gene from *Solanum tuberosum* cv. Kardal in  
5 the reversed orientation under control of the tuber specific patatin  
promoter. The construction of this vector is very similar to the  
construction of pMOG845 and can be performed by any person skilled in the  
art. After mobilization of this binary vector by triparental mating to  
*Agrobacterium*, this strain can be used in potato transformation experiments  
10 to generate transgenic plants having reduced levels of trehalase activity  
in tuber-tissue.

#### **EXAMPLE 1**

##### Cloning of a full length *E. coli* otsA gene

15 In *E. coli* trehalose phosphate synthase (TPS) is encoded by the  
*otsA* gene located in the operon *otsBA*. The cloning and sequence  
determination of the *otsA* gene is described in detail in Example I of  
PCT/EP94/02167, herein incorporated by reference. To effectuate its  
expression in plant cells, the open reading frame has been linked to the  
20 transcriptional regulatory elements of the CaMV 35S RNA promoter, the  
translational enhancer of the ALMV leader, and the transcriptional  
terminator of the NOS-gene, as described in greater detail in Example I of  
PCT/EP94/02167.

A binary vector, pMOG799 (Fig. 1), containing the plant expressible *otsA*  
25 gene and a the kanamycin resistance gene as selectable marker between T-DNA  
borders, is used to transform potato and tobacco.

#### **EXAMPLE 2**

##### Trehalose production in tobacco plants transformed with pMOG799

30 Tobacco leaf discs are transformed with the binary vector pMOG799 using  
*Agrobacterium tumefaciens*. Transgenic shoots are selected on kanamycin.  
Transgenic plants are transferred to the greenhouse to flower and set seed  
after selfing (S1). Seeds of these transgenic plants are surface sterilised  
and germinated *in vitro* on medium with Kanamycin. Kanamycin resistant  
35 seedlings and wild-type tobacco plants are transferred to MS-medium  
supplemented with  $10^{-3}$  M Validamycin A. As a control, transgenic seedlings

and wild-type plants are transferred to medium without Validamycin A. Analysis of leaves and roots of plants grown on Validamycin A shows elevated levels of trehalose compared to the control plants (Table 1). No trehalose was detected in wild-type tobacco plants.

5

Table 1

	with Validamycin A		without Validamycin A	
	leaf	roots	leaf	roots
pMOG799.1	0.0081	0.0044	-	0.003
10 pMOG799.13	0.0110	0.0080	-	-
pMOG799.31	0.0008	0.0088	-	-
Wild-type SR1	-	-	-	-

**EXAMPLE 3**

15 Trehalose production in potato micro-tubers transformed with pMOG845  
 Potato *Solanum tuberosum* cv. Kardal tuber discs are transformed with *Agrobacterium tumefaciens* EHA105 harbouring the binary vector pMOG845. Transgenic shoots are selected on kanamycin. Micro-tubers (m-tubers) are induced on stem segments of transgenic and wild-type plants cultured on m-tuber inducing medium supplemented with  $10^{-3}$  M Validamycin A. As a control, m-tubers are induced on medium without Validamycin A. M-tubers induced on medium with Validamycin A showed elevated levels of trehalose in comparison with m-tubers grown on medium without Validamycin A (Table 2). No trehalose was detected in wild-type m-tubers.

25

Table 2.

	Trehalose (% fresh weight)	
	+Validamycin A	-Validamycin A
845-2	0.016	-
30 845-4	-	-
845-8	0.051	-
845-13	0.005	-
845-22	0.121	-
845-25	0.002	-
35 wT Kardal	-	-

## EXAMPLE 4

Trehalose production in hydrocultures of tobacco plants transformed with pMOG799

Seeds (S1) of selfed tobacco plants transformed with the binary vector pMOG799 are surface sterilised and germinated in vitro on MS20MS medium containing 50 µg/ml Kanamycin. Kanamycin resistant seedlings are transferred to soil and grown in a growth chamber (temp. 23°C, 16 hours of light/day). After four weeks, seedlings were transferred to hydrocultures with ASEF clay beads with approximately 450 ml of medium. The medium contains 40 g/l Solacol dissolved in nano-water buffered with 0.5 g/l MES to adjust to pH 6.0 which is sieved through a filter to remove solid particles. Essential salts are supplemented by adding POKONTM (1.5 ml/l). The following antibiotics are added to prevent growth of micro-organisms: 500µg/ml Carbenicillin, 40µg/ml Nystatin and 100µg/ml Vancomycin. As a control, transgenic seedlings and wild-type plants are transferred to medium without Solacol. Analysis of leaves of plants grown on Solacol shows elevated levels of trehalose compared to the control plants (Table 3). No trehalose was detected in wild-type tobacco plants.

20 Table 3

	Solacol	Trehalose (%w/w)
pMOG 799.1-1	+	0.008
pMOG 799.1-2	+	0.004
pMOG 799.1-3	-	-
25 pMOG 799.1-4	-	-
pMOG 799.1-5	+	0.008
pMOG 799.1-6	-	-
pMOG 799.1-7	+	0.005
pMOG 799.1-8	-	-
30 pMOG 799.1-9	-	-
pMOG 799.1-10	+	0.007
Wild-type SR1-1	-	-
Wild-type SR1-2	+	-
35 Wild-type SR1-3	-	-
Wild-type SR1-4	+	-

**Example 5**Cloning of a full length cDNA encoding trehalase from potato tuber tissue

Using the amino acid sequence of the conserved regions of known trehalase genes (*E.coli*, Yeast, Rabbit, *B. mori*) (figure 4), four degenerated primers were designed:

```

10      C   C C   CGT   GT A   TTAT
      GG GGI G TT IGA T TA TGGGAC      Tase24 (SEQIDNO:11)
       T   A A   TAA AG C   CGGC

      TAA   GT
15      GTICCI GGIGGICGITT IGA T
       CGT   AG

      T   GA   TG   A   A
20      GGIGG TGI CT ICGI CA IAG TA TA
       C           G   G

      C G   AT   A
      I C TTI CCATCC AAICCTC
       G A   GC   G

```

Tase25 (SEQIDNO:12)

Tase26 (SEQIDNO:13)

Tase27 (SEQIDNO:14)

25

Combinations of these primers in PCR experiments with genomic DNA and cDNA from *S. tuberosum* cv. Kardal leaf and tuber material respectively as template, resulted in several fragments of the expected length. A number of 190 bp. fragments obtained with the primer combination Tase24 and Tase 26 were subcloned into a pGEM T vector and sequenced. Several of the clones analyzed showed homology with known trehalase sequences. To exclude the isolation of non-plant derived trehalase sequences, Southern blot analysis was performed with gDNA from potato cv. Kardal. A number of clones isolated did not cross-hybridize with Kardal genomic DNA and were discarded. Two isolated clones were identical, gTase15.4 derived from a genomic PCR experiment and cTase5.2 derived from a PCR on cDNA, both showing hybridization in Southern blot analysis. One single hybridizing band was detected (EcoRI 1.5 Kb, HindIII 3 Kb and BamHI larger than 12 Kb) suggesting the presence of only one copy of the isolated PCR fragment.

A cDNA library was constructed out of poly A<sup>+</sup> RNA from potato tubers (cv. Kardal) using a Stratagene cDNA synthesis kit and the vector Lambda ZAPII. Recombinant phages (500.000) were screened with the radiolabeled cTase5.2 PCR fragment resulting in the identification of 3 positive clones. After purification, two clones were characterised with restriction enzymes revealing inserts of 2.15 and 2.3 kb respectively. Their nucleotide sequence was 100% identical. The nucleic acid sequence

of one of these trehalase cDNA clones from *Solanum tuberosum* including its open reading frame is depicted in seq ID no:9, while the aminoacid sequence derived from this nucleic acid sequence is shown in seq. ID no:10. A plasmid harbouring an insert comprising the genetic information  
5 coding for trehalase has been deposited under no. CBS 804.95 with the Centraal Bureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, the Netherlands on December 8, 1995.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT:  
(A) NAME: MOGEN International n.v.  
(B) STREET: Einsteinweg 97  
(C) CITY: LEIDEN  
(D) STATE: Zuid-Holland  
10 (E) COUNTRY: The Netherlands  
(F) POSTAL CODE (ZIP): NL-2333 CB  
(G) TELEPHONE: (31).(71).5258282  
(H) TELEFAX: (31).(71).5221471
- 15 (ii) TITLE OF INVENTION: Enhanced accumulation of trehalose in plants
- (iii) NUMBER OF SEQUENCES: 14
- (iv) COMPUTER READABLE FORM:  
20 (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)
- 25 (vi) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: EP 95.200.008.1  
(B) FILING DATE: 04-JAN-1995
- (vi) PRIOR APPLICATION DATA:  
30 (A) APPLICATION NUMBER: EP 95.202.415.6  
(B) FILING DATE: 07-SEP-1995
- (2) INFORMATION FOR SEQ ID NO: 1:  
35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1446 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
40 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO  
45 (iii) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:  
(A) ORGANISM: Escherichia coli  
50 (B) STRAIN: CLONE: 7F11
- (viii) POSITION IN GENOME:  
(B) MAP POSITION: 41-42'

27

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 19..1446

(D) OTHER INFORMATION: /product= "trehalose phosphate  
synthase"  
/gene= "otsA"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

10	GAGAAAATAA CAGGAGTG ATG ACT ATG AGT CGT TTA GTC GTA GTA TCT AAC	51
	Met Thr Met Ser Arg Leu Val Val Val Ser Asn	
	1 5 10	
15	CGG ATT GCA CCA CCA GAC GAG CAC GCC GCC AGT GCC GGT GGC CTT GCC	99
	Arg Ile Ala Pro Pro Asp Glu His Ala Ala Ser Ala Gly Gly Leu Ala	
	15 20 25	
20	GTT GGC ATA CTG GGG GCA CTG AAA GCC GCA GGC GGA CTG TGG TTT GGC	147
	Val Gly Ile Leu Gly Ala Leu Lys Ala Ala Gly Gly Leu Trp Phe Gly	
	30 35 40	
25	TGG AGT GGT GAA ACA GGG AAT GAG GAT CAG CCG CTA AAA AAG GTG AAA	195
	Trp Ser Gly Glu Thr Gly Asn Glu Asp Gln Pro Leu Lys Lys Val Lys	
	45 50 55	
30	AAA GGT AAC ATT ACG TGG GCC TCT TTT AAC CTC AGC GAA CAG GAC CTT	243
	Lys Gly Asn Ile Thr Trp Ala Ser Phe Asn Leu Ser Glu Gln Asp Leu	
	60 65 70 75	
35	GAC GAA TAC TAC AAC CAA TTC TCC AAT GCC GTT CTC TGG CCC GCT TTT	291
	Asp Glu Tyr Tyr Asn Gln Phe Ser Asn Ala Val Leu Trp Pro Ala Phe	
	80 85 90	
40	CAT TAT CGG CTC GAT CTG GTG CAA TTT CAG CGT CCT GCC TGG GAC GGC	339
	His Tyr Arg Leu Asp Leu Val Gln Phe Gln Arg Pro Ala Trp Asp Gly	
	95 100 105	
45	TAT CTA CGC GTA AAT GCG TTG CTG GCA GAT AAA TTA CTG CCG CTG TTG	387
	Tyr Leu Arg Val Asn Ala Leu Leu Ala Asp Lys Leu Leu Pro Leu Leu	
	110 115 120	
50	CAA GAC GAT GAC ATT ATC TGG ATC CAC GAT TAT CAC CTG TTG CCA TTT	435
	Gln Asp Asp Asp Ile Ile Trp Ile His Asp Tyr His Leu Leu Pro Phe	
	125 130 135	
55	GCG CAT GAA TTA CGC AAA CGG GGA GTG AAT AAT CGC ATT GGT TTC TTT	483
	Ala His Glu Leu Arg Lys Arg Gly Val Asn Asn Arg Ile Gly Phe Phe	
	140 145 150 155	
60	CTG CAT ATT CCT TTC CCG ACA CCG GAA ATC TTC AAC GCG CTG CCG ACA	531
	Leu His Ile Pro Phe Pro Thr Pro Glu Ile Phe Asn Ala Leu Pro Thr	
	160 165 170	

28

	TAT	GAC	ACC	TTG	CTT	GAA	CAG	CTT	TGT	GAT	TAT	GAT	TTG	CTG	GGT	TTC	579
	Tyr	Asp	Thr	Leu	Leu	Glu	Gln	Leu	Cys	Asp	Tyr	Asp	Leu	Leu	Gly	Phe	
				175					180					185			
5	CAG	ACA	GAA	AAC	GAT	CGT	CTG	GCG	TTC	CTG	GAT	TGT	CTT	TCT	AAC	CTG	627
	Gln	Thr	Glu	Asn	Asp	Arg	Leu	Ala	Phe	Leu	Asp	Cys	Leu	Ser	Asn	Leu	
			190					195					200				
	ACC	CGC	GTC	ACG	ACA	CGT	AGC	GCA	AAA	AGC	CAT	ACA	GCC	TGG	GGC	AAA	675
10	Thr	Arg	Val	Thr	Thr	Arg	Ser	Ala	Lys	Ser	His	Thr	Ala	Trp	Gly	Lys	
		205					210					215					
	GCA	TTT	CGA	ACA	GAA	GTC	TAC	CCG	ATC	GGC	ATT	GAA	CCG	AAA	GAA	ATA	723
	Ala	Phe	Arg	Thr	Glu	Val	Tyr	Pro	Ile	Gly	Ile	Glu	Pro	Lys	Glu	Ile	
15	220					225					230				235		
	GCC	AAA	CAG	GCT	GCC	GGG	CCA	CTG	CCG	CCA	AAA	CTG	GCG	CAA	CTT	AAA	771
	Ala	Lys	Gln	Ala	Ala	Gly	Pro	Leu	Pro	Pro	Lys	Leu	Ala	Gln	Leu	Lys	
				240					245					250			
20	GCG	GAA	CTG	AAA	AAC	GTA	CAA	AAT	ATC	TTT	TCT	GTC	GAA	CGG	CTG	GAT	819
	Ala	Glu	Leu	Lys	Asn	Val	Gln	Asn	Ile	Phe	Ser	Val	Glu	Arg	Leu	Asp	
				255				260					265				
25	TAT	TCC	AAA	GGT	TTG	CCA	GAG	CGT	TTT	CTC	GCC	TAT	GAA	GCG	TTG	CTG	867
	Tyr	Ser	Lys	Gly	Leu	Pro	Glu	Arg	Phe	Leu	Ala	Tyr	Glu	Ala	Leu	Leu	
			270				275					280					
	GAA	AAA	TAT	CCG	CAG	CAT	CAT	GGT	AAA	ATT	CGT	TAT	ACC	CAG	ATT	GCA	915
30	Glu	Lys	Tyr	Pro	Gln	His	His	Gly	Lys	Ile	Arg	Tyr	Thr	Gln	Ile	Ala	
		285					290				295						
	CCA	ACG	TCG	CGT	GGT	GAT	GTG	CAA	GCC	TAT	CAG	GAT	ATT	CGT	CAT	CAG	963
	Pro	Thr	Ser	Arg	Gly	Asp	Val	Gln	Ala	Tyr	Gln	Asp	Ile	Arg	His	Gln	
35	300					305					310					315	
	CTC	GAA	AAT	GAA	GCT	GGA	CGA	ATT	AAT	GGT	AAA	TAC	GGG	CAA	TTA	GGC	1011
	Leu	Glu	Asn	Glu	Ala	Gly	Arg	Ile	Asn	Gly	Lys	Tyr	Gly	Gln	Leu	Gly	
				320					325					330			
40	TGG	ACG	CCG	CTT	TAT	TAT	TTG	AAT	CAG	CAT	TTT	GAC	CGT	AAA	TTA	CTG	1059
	Trp	Thr	Pro	Leu	Tyr	Tyr	Leu	Asn	Gln	His	Phe	Asp	Arg	Lys	Leu	Leu	
				335				340					345				
45	ATG	AAA	ATA	TTC	CGC	TAC	TCT	GAC	GTG	GGC	TTA	GTG	ACG	CCA	CTG	CGT	1107
	Met	Lys	Ile	Phe	Arg	Tyr	Ser	Asp	Val	Gly	Leu	Val	Thr	Pro	Leu	Arg	
			350				355					360					
	GAC	GGG	ATG	AAC	CTG	GTA	GCA	AAA	GAG	TAT	GTT	GCT	GCT	CAG	GAC	CCA	1155
50	Asp	Gly	Met	Asn	Leu	Val	Ala	Lys	Glu	Tyr	Val	Ala	Ala	Gln	Asp	Pro	
		365					370					375					



29

	GCC AAT CCG GGC GTT CTT GTT CTT TCG CAA TTT GCG GGA GCG GCA AAC	1203
	Ala Asn Pro Gly Val Leu Val Leu Ser Gln Phe Ala Gly Ala Ala Asn	
	380 385 390 395	
5	GAG TTA ACG TCG GCG TTA ATT GTT AAC CCC TAC GAT CGT GAC GAA GTT	1251
	Glu Leu Thr Ser Ala Leu Ile Val Asn Pro Tyr Asp Arg Asp Glu Val	
	400 405 410	
10	GCA GCT GCG CTG GAT CGT GCA TTG ACT ATG TCG CTG GCG GAA CGT ATT	1299
	Ala Ala Ala Leu Asp Arg Ala Leu Thr Met Ser Leu Ala Glu Arg Ile	
	415 420 425	
15	TCC CGT CAT GCA GAA ATG CTG GAC GTT ATC GTG AAA AAC GAT ATT AAC	1347
	Ser Arg His Ala Glu Met Leu Asp Val Ile Val Lys Asn Asp Ile Asn	
	430 435 440	
20	CAC TGG CAG GAG TGC TTC ATT AGC GAC CTA AAG CAG ATA GTT CCG CGA	1395
	His Trp Gln Glu Cys Phe Ile Ser Asp Leu Lys Gln Ile Val Pro Arg	
	445 450 455	
25	AGC GCG GAA AGC CAG CAG CGC GAT AAA GTT GCT ACC TTT CCA AAG CTT	1443
	Ser Ala Glu Ser Gln Gln Arg Asp Lys Val Ala Thr Phe Pro Lys Leu	
	460 465 470 475	
30	GCG	1446
	Ala	

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 476 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

40	Met Thr Met Ser Arg Leu Val Val Val Ser Asn Arg Ile Ala Pro Pro
	1 5 10 15
	Asp Glu His Ala Ala Ser Ala Gly Gly Leu Ala Val Gly Ile Leu Gly
	20 25 30
45	Ala Leu Lys Ala Ala Gly Gly Leu Trp Phe Gly Trp Ser Gly Glu Thr
	35 40 45
50	Gly Asn Glu Asp Gln Pro Leu Lys Lys Val Lys Lys Gly Asn Ile Thr
	50 55 60
	Trp Ala Ser Phe Asn Leu Ser Glu Gln Asp Leu Asp Glu Tyr Tyr Asn
	65 70 75 80

30

	Gln	Phe	Ser	Asn	Ala	Val	Leu	Trp	Pro	Ala	Phe	His	Tyr	Arg	Leu	Asp
					85					90						95
5	Leu	Val	Gln	Phe	Gln	Arg	Pro	Ala	Trp	Asp	Gly	Tyr	Leu	Arg	Val	Asn
				100					105					110		
	Ala	Leu	Leu	Ala	Asp	Lys	Leu	Leu	Pro	Leu	Leu	Gln	Asp	Asp	Asp	Ile
			115					120					125			
10	Ile	Trp	Ile	His	Asp	Tyr	His	Leu	Leu	Pro	Phe	Ala	His	Glu	Leu	Arg
		130					135					140				
	Lys	Arg	Gly	Val	Asn	Asn	Arg	Ile	Gly	Phe	Phe	Leu	His	Ile	Pro	Phe
	145					150					155					160
15	Pro	Thr	Pro	Glu	Ile	Phe	Asn	Ala	Leu	Pro	Thr	Tyr	Asp	Thr	Leu	Leu
					165					170					175	
20	Glu	Gln	Leu	Cys	Asp	Tyr	Asp	Leu	Leu	Gly	Phe	Gln	Thr	Glu	Asn	Asp
				180					185					190		
	Arg	Leu	Ala	Phe	Leu	Asp	Cys	Leu	Ser	Asn	Leu	Thr	Arg	Val	Thr	Thr
			195					200					205			
25	Arg	Ser	Ala	Lys	Ser	His	Thr	Ala	Trp	Gly	Lys	Ala	Phe	Arg	Thr	Glu
		210					215					220				
	Val	Tyr	Pro	Ile	Gly	Ile	Glu	Pro	Lys	Glu	Ile	Ala	Lys	Gln	Ala	Ala
	225					230					235					240
30	Gly	Pro	Leu	Pro	Pro	Lys	Leu	Ala	Gln	Leu	Lys	Ala	Glu	Leu	Lys	Asn
					245					250					255	
	Val	Gln	Asn	Ile	Phe	Ser	Val	Glu	Arg	Leu	Asp	Tyr	Ser	Lys	Gly	Leu
				260					265					270		
35	Pro	Glu	Arg	Phe	Leu	Ala	Tyr	Glu	Ala	Leu	Leu	Glu	Lys	Tyr	Pro	Gln
			275					280					285			
40	His	His	Gly	Lys	Ile	Arg	Tyr	Thr	Gln	Ile	Ala	Pro	Thr	Ser	Arg	Gly
		290					295					300				
	Asp	Val	Gln	Ala	Tyr	Gln	Asp	Ile	Arg	His	Gln	Leu	Glu	Asn	Glu	Ala
	305					310					315					320
45	Gly	Arg	Ile	Asn	Gly	Lys	Tyr	Gly	Gln	Leu	Gly	Trp	Thr	Pro	Leu	Tyr
					325					330					335	
	Tyr	Leu	Asn	Gln	His	Phe	Asp	Arg	Lys	Leu	Leu	Met	Lys	Ile	Phe	Arg
				340					345					350		
50	Tyr	Ser	Asp	Val	Gly	Leu	Val	Thr	Pro							

31

Val Ala Lys Glu Tyr Val Ala Ala Gln Asp Pro Ala Asn Pro Gly Val  
 370 375 380

5 Leu Val Leu Ser Gln Phe Ala Gly Ala Ala Asn Glu Leu Thr Ser Ala  
 385 390 395 400

Leu Ile Val Asn Pro Tyr Asp Arg Asp Glu Val Ala Ala Ala Leu Asp  
 405 410 415

10 Arg Ala Leu Thr Met Ser Leu Ala Glu Arg Ile Ser Arg His Ala Glu  
 420 425 430

Met Leu Asp Val Ile Val Lys Asn Asp Ile Asn His Trp Gln Glu Cys  
 435 440 445

15 Phe Ile Ser Asp Leu Lys Gln Ile Val Pro Arg Ser Ala Glu Ser Gln  
 450 455 460

Gln Arg Asp Lys Val Ala Thr Phe Pro Lys Leu Ala  
 20 465 470 475

## (2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
- 25 (A) LENGTH: 25 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- 30 (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: YES
- (iii) ANTI-SENSE: NO
- 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AAGCTTATGT TGCCATATAG AGTAG

25

## 40 (2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
- 45 (A) LENGTH: 24 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- 50 (iii) HYPOTHETICAL: YES
- (iii) ANTI-SENSE: NO

32

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GTAGTTGCCA TGGTGCAAAT GTTC

24

5 (2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- 10 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15 (iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

20 AGCTCTGCAG TGAGGTACCA

20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GACGTCCTC CATGGTTCGA

20

(2) INFORMATION FOR SEQ ID NO: 7:

40

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- 45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTACCCTGCA GTGTGACCCT AGAC

24

33

## (2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

10

## (iii) HYPOTHETICAL: YES

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

15

TCGATTCATA GAAGCTTAGA T

21

## (2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

20

- (A) LENGTH: 2207 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

25

## (ii) MOLECULE TYPE: cDNA to mRNA

## (iii) HYPOTHETICAL: NO

30

## (iii) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Solanum tuberosum  
(B) STRAIN: Kardal

35

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
(B) LOCATION: 161..1906

40

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature  
(B) LOCATION: 842..850  
(D) OTHER INFORMATION: /function= "putative  
glycosylationsite"

45

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTTTTCTGAG TAATAACATA GGCATTGATT TTTTTCAT TAATAACACC TGCAAACATT

60

50 CCCATTGCCG GCATTCTCTG TTCTTACAAA AAAAAACATT TTTTGTTC CATAAATTAG

120

TTATGGCATC AGTATTGAAC CCTTTAACTT GTTATACAAT ATG GGT AAA GCT ATA  
Met Gly Lys Ala Ile

175

1

5

34

	ATT	TTT	ATG	ATT	TTT	ACT	ATG	TCT	ATG	AAT	ATG	ATT	AAA	GCT	GAA	ACT	223
	Ile	Phe	Met	Ile	Phe	Thr	Met	Ser	Met	Asn	Met	Ile	Lys	Ala	Glu	Thr	
					10					15					20		
5	TGC	AAA	TCC	ATT	GAT	AAG	GGT	CCT	GTA	ATC	CCA	ACA	ACC	CCT	TTA	GTG	271
	Cys	Lys	Ser	Ile	Asp	Lys	Gly	Pro	Val	Ile	Pro	Thr	Thr	Pro	Leu	Val	
				25				30						35			
10	ATT	TTT	CTT	GAA	AAA	GTT	CAA	GAA	GCT	GCT	CTT	CAA	ACT	TAT	GGC	CAT	319
	Ile	Phe	Leu	Glu	Lys	Val	Gln	Glu	Ala	Ala	Leu	Gln	Thr	Tyr	Gly	His	
			40					45					50				
15	AAA	GGG	TTT	GAT	GCT	AAA	CTG	TTT	GTT	GAT	ATG	TCA	CTG	AGA	GAG	AGT	367
	Lys	Gly	Phe	Asp	Ala	Lys	Leu	Phe	Val	Asp	Met	Ser	Leu	Arg	Glu	Ser	
		55					60					65					
20	CTT	TCA	GAA	ACA	GTT	GAA	GCT	TTT	AAT	AAG	CTT	CCA	AGA	GTT	GTG	AAT	415
	Leu	Ser	Glu	Thr	Val	Glu	Ala	Phe	Asn	Lys	Leu	Pro	Arg	Val	Val	Asn	
	70					75					80					85	
25	GGT	TCA	ATA	TCA	AAA	AGT	GAT	TTG	GAT	GGT	TTT	ATA	GGT	AGT	TAC	TTG	463
	Gly	Ser	Ile	Ser	Lys	Ser	Asp	Leu	Asp	Gly	Phe	Ile	Gly	Ser	Tyr	Leu	
					90					95					100		
30	AGT	AGT	CCT	GAT	AAG	GAT	TTG	GTT	TAT	GTT	GAG	CCT	ATG	GAT	TTT	GTG	511
	Ser	Ser	Pro	Asp	Lys	Asp	Leu	Val	Tyr	Val	Glu	Pro	Met	Asp	Phe	Val	
				105					110					115			
35	GCT	GAG	CCT	GAA	GGC	TTT	TTG	CCA	AAG	GTG	AAG	AAT	TCT	GAG	GTG	AGG	559
	Ala	Glu	Pro	Glu	Gly	Phe	Leu	Pro	Lys	Val	Lys	Asn	Ser	Glu	Val	Arg	
			120					125					130				
40	GCA	TGG	GCA	TTG	GAG	GTG	CAT	TCA	CTT	TGG	AAG	AAT	TTA	AGT	AGG	AAA	607
	Ala	Trp	Ala	Leu	Glu	Val	His	Ser	Leu	Trp	Lys	Asn	Leu	Ser	Arg	Lys	
		135					140					145					
45	GTG	GCT	GAT	CAT	GTA	TTG	GAA	AAA	CCA	GAG	TTG	TAT	ACT	TTG	CTT	CCA	655
	Val	Ala	Asp	His	Val	Leu	Glu	Lys	Pro	Glu	Leu	Tyr	Thr	Leu	Leu	Pro	
	150					155					160				165		
50	TTG	AAA	AAT	CCA	GTT	ATT	ATA	CCG	GGA	TCG	CGT	TTT	AAG	GAG	GTT	TAT	703
	Leu	Lys	Asn	Pro	Val	Ile	Ile	Pro	Gly	Ser	Arg	Phe	Lys	Glu	Val	Tyr	
				170						175					180		
55	TAT	TGG	GAT	TCT	TAT	TGG	GTA	ATA	AGG	GGT	TTG	TTA	GCA	AGC	AAA	ATG	751
	Tyr	Trp	Asp	Ser	Tyr	Trp	Val	Ile	Arg	Gly	Leu	Leu	Ala	Ser	Lys	Met	
				185					190					195			
60	TAT	GAA	ACT	GCA	AAA	GGG	ATT	GTG	ACT	AAT	CTG	GTT	TCT	CTG	ATA	GAT	799
	Tyr	Glu	Thr	Ala	Lys	Gly	Ile	Val	Thr	Asn	Leu	Val	Ser	Leu	Ile	Asp	
			200					205						210			

35

	CAA	TTT	GGT	TAT	GTT	CTT	AAC	GGT	GCA	AGA	GCA	TAC	TAC	AGT	AAC	AGA	847
	Gln	Phe	Gly	Tyr	Val	Leu	Asn	Gly	Ala	Arg	Ala	Tyr	Tyr	Ser	Asn	Arg	
	215						220					225					
5	AGT	CAG	CCT	CCT	GTC	CTG	GCC	ACG	ATG	ATT	GTT	GAC	ATA	TTC	AAT	CAG	895
	Ser	Gln	Pro	Pro	Val	Leu	Ala	Thr	Met	Ile	Val	Asp	Ile	Phe	Asn	Gln	
	230					235					240					245	
10	ACA	GGT	GAT	TTA	AAT	TTG	GTT	AGA	AGA	TCC	CTT	CCT	GCT	TTG	CTC	AAG	943
	Thr	Gly	Asp	Leu	Asn	Leu	Val	Arg	Arg	Ser	Leu	Pro	Ala	Leu	Leu	Lys	
					250					255					260		
15	GAG	AAT	CAT	TTT	TGG	AAT	TCA	GGA	ATA	CAT	AAG	GTG	ACT	ATT	CAA	GAT	991
	Glu	Asn	His	Phe	Trp	Asn	Ser	Gly	Ile	His	Lys	Val	Thr	Ile	Gln	Asp	
				265					270					275			
20	GCT	CAG	GGA	TCA	AAC	CAC	AGC	TTG	AGT	CGG	TAC	TAT	GCT	ATG	TGG	AAT	1039
	Ala	Gln	Gly	Ser	Asn	His	Ser	Leu	Ser	Arg	Tyr	Tyr	Ala	Met	Trp	Asn	
			280					285					290				
	AAG	CCC	CGT	CCA	GAA	TCG	TCA	ACT	ATA	GAC	AGT	GAA	ACA	GCT	TCC	GTA	1087
	Lys	Pro	Arg	Pro	Glu	Ser	Ser	Thr	Ile	Asp	Ser	Glu	Thr	Ala	Ser	Val	
	295						300					305					
25	CTC	CCA	AAT	ATA	TGT	GAA	AAA	AGA	GAA	TTA	TAC	CGT	GAA	CTG	GCA	TCA	1135
	Leu	Pro	Asn	Ile	Cys	Glu	Lys	Arg	Glu	Leu	Tyr	Arg	Glu	Leu	Ala	Ser	
	310					315					320					325	
30	GCT	GCT	GAA	AGT	GGA	TGG	GAT	TTC	AGT	TCA	AGA	TGG	ATG	AGC	AAC	GGA	1183
	Ala	Ala	Glu	Ser	Gly	Trp	Asp	Phe	Ser	Ser	Arg	Trp	Met	Ser	Asn	Gly	
					330					335					340		
35	TCT	GAT	CTG	ACA	ACA	ACT	AGT	ACA	ACA	TCA	ATT	CTA	CCA	GTT	GAT	TTG	1231
	Ser	Asp	Leu	Thr	Thr	Thr	Ser	Thr	Thr	Ser	Ile	Leu	Pro	Val	Asp	Leu	
				345					350					355			
40	AAT	GCA	TTC	CTT	CTG	AAG	ATG	GAA	CTT	GAC	ATT	GCC	TTT	CTA	GCA	AAT	1279
	Asn	Ala	Phe	Leu	Leu	Lys	Met	Glu	Leu	Asp	Ile	Ala	Phe	Leu	Ala	Asn	
			360					365					370				
	CTT	GTT	GGA	GAA	AGT	AGC	ACG	GCT	TCA	CAT	TTT	ACA	GAA	GCT	GCT	CAA	1327
	Leu	Val	Gly	Glu	Ser	Ser	Thr	Ala	Ser	His	Phe	Thr	Glu	Ala	Ala	Gln	
			375				380					385					
45	AAT	AGA	CAG	AAG	GCT	ATA	AAC	TGT	ATC	TTT	TGG	AAC	GCA	GAG	ATG	GGG	1375
	Asn	Arg	Gln	Lys	Ala	Ile	Asn	Cys	Ile	Phe	Trp	Asn	Ala	Glu	Met	Gly	
	390					395					400					405	
50	CAA	TGG	CTT	GAT	TAC	TGG	CTT	ACC	AAC	AGC	GAC	ACA	TCT	GAG	GAT	ATT	1423
	Gln	Trp	Leu	Asp	Tyr	Trp	Leu	Thr	Asn	Ser	Asp	Thr	Ser	Glu	Asp	Ile	
					410					415					420		

36

	TAT AAA TGG GAA GAT TTG CAC CAG AAC AAG AAG TCA TTT GCC TCT AAT	1471
	Tyr Lys Trp Glu Asp Leu His Gln Asn Lys Lys Ser Phe Ala Ser Asn	
	425 430 435	
5	TTT GTT CCG CTG TGG ACT GAA ATT TCT TGT TCA GAT AAT AAT ATC ACA	1519
	Phe Val Pro Leu Trp Thr Glu Ile Ser Cys Ser Asp Asn Asn Ile Thr	
	440 445 450	
10	ACT CAG AAA GTA GTT CAA AGT CTC ATG AGC TCG GGC TTG CTT CAG CCT	1567
	Thr Gln Lys Val Val Gln Ser Leu Met Ser Ser Gly Leu Leu Gln Pro	
	455 460 465	
15	GCA GGG ATT GCA ATG ACC TTG TCT AAT ACT GGA CAG CAA TGG GAT TTT	1615
	Ala Gly Ile Ala Met Thr Leu Ser Asn Thr Gly Gln Gln Trp Asp Phe	
	470 475 480 485	
20	CCG AAT GGT TGG CCC CCC CTT CAA CAC ATA ATC ATT GAA GGT CTC TTA	1663
	Pro Asn Gly Trp Pro Pro Leu Gln His Ile Ile Ile Glu Gly Leu Leu	
	490 495 500	
25	AGG TCT GGA CTA GAA GAG GCA AGA ACC TTA GCA AAA GAC ATT GCT ATT	1711
	Arg Ser Gly Leu Glu Glu Ala Arg Thr Leu Ala Lys Asp Ile Ala Ile	
	505 510 515	
30	CGC TGG TTA AGA ACT AAC TAT GTG ACT TAC AAG AAA ACC GGT GCT ATG	1759
	Arg Trp Leu Arg Thr Asn Tyr Val Thr Tyr Lys Lys Thr Gly Ala Met	
	520 525 530	
35	TAT GAA AAA TAT GAT GTC ACA AAA TGT GGA GCA TAT GGA GGT GGT GGT	1807
	Tyr Glu Lys Tyr Asp Val Thr Lys Cys Gly Ala Tyr Gly Gly Gly Gly	
	535 540 545	
40	GAA TAT ATG TCC CAA ACG GGT TTC GGA TGG TCA AAT GGC GTT GTA CTG	1855
	Glu Tyr Met Ser Gln Thr Gly Phe Gly Trp Ser Asn Gly Val Val Leu	
	550 555 560 565	
45	GCA CTT CTA GAG GAA TTT GGA TGG CCT GAA GAT TTG AAG ATT GAT TGC	1903
	Ala Leu Leu Glu Glu Phe Gly Trp Pro Glu Asp Leu Lys Ile Asp Cys	
	570 575 580	
50	TAATGAGCAA GTAGAAAAGC CAAATGAAAC ATCATTGAGT TTTATTTTCT TCTTTTGTTA	1963
	AAATAAGCTG CAATGGTTTG CTGATAGTTT ATGTTTTGTA TTACTATTTC ATAAGGTTTT	2023
	TGTACCATAT CAAGTGATAT TACCATGAAC TATGTCGTTT GGACTCTTCA AATCGGATTT	2083
	TGCAAAAATA ATGCAGTTTT GGAGAATCCG ATAACATAGA CCATGTATGG ATCTAAATTG	2143
	TAAACAGCTT ACTATATTAA GTAAAAGAAA GATGATTCCT CTGCTTTAAA AAAAAAAAAA	2203
	AAAA	2207



37

## (2) INFORMATION FOR SEQ ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 581 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

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Met Gly Lys Ala Ile Ile Phe Met Ile Phe Thr Met Ser Met Asn Met
 1             5             10             15
15 Ile Lys Ala Glu Thr Cys Lys Ser Ile Asp Lys Gly Pro Val Ile Pro
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    Thr Thr Pro Leu Val Ile Phe Leu Glu Lys Val Gln Glu Ala Ala Leu
      35             40             45
20 Gln Thr Tyr Gly His Lys Gly Phe Asp Ala Lys Leu Phe Val Asp Met
      50             55             60
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25 65             70             75             80
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      130            135            140
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    Tyr Thr Leu Leu Pro Leu Lys Asn Pro Val Ile Ile Pro Gly Ser Arg
      165            170            175
45 Phe Lys Glu Val Tyr Tyr Trp Asp Ser Tyr Trp Val Ile Arg Gly Leu
      180            185            190
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Tyr Tyr Ser Asn Arg Ser Gln Pro Pro Val Leu Ala Thr Met Ile Val  
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 290 295 300  
 15 Glu Thr Ala Ser Val Leu Pro Asn Ile Cys Glu Lys Arg Glu Leu Tyr  
 305 310 315 320  
 Arg Glu Leu Ala Ser Ala Ala Glu Ser Gly Trp Asp Phe Ser Ser Arg  
 325 330 335  
 20 Trp Met Ser Asn Gly Ser Asp Leu Thr Thr Thr Ser Thr Thr Ser Ile  
 340 345 350  
 25 Leu Pro Val Asp Leu Asn Ala Phe Leu Leu Lys Met Glu Leu Asp Ile  
 355 360 365  
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 370 375 380  
 30 Thr Glu Ala Ala Gln Asn Arg Gln Lys Ala Ile Asn Cys Ile Phe Trp  
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 Asn Ala Glu Met Gly Gln Trp Leu Asp Tyr Trp Leu Thr Asn Ser Asp  
 405 410 415  
 35 Thr Ser Glu Asp Ile Tyr Lys Trp Glu Asp Leu His Gln Asn Lys Lys  
 420 425 430  
 40 Ser Phe Ala Ser Asn Phe Val Pro Leu Trp Thr Glu Ile Ser Cys Ser  
 435 440 445  
 Asp Asn Asn Ile Thr Thr Gln Lys Val Val Gln Ser Leu Met Ser Ser  
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 45 Gly Leu Leu Gln Pro Ala Gly Ile Ala Met Thr Leu Ser Asn Thr Gly  
 465 470 475 480  
 Gln Gln Trp Asp Phe Pro Asn Gly Trp Pro Pro Leu Gln His Ile Ile  
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 50 Ile Glu Gly Leu Leu Arg Ser Gly Leu Glu Glu Ala Arg Thr Leu Ala  
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39

Lys Asp Ile Ala Ile Arg Trp Leu Arg Thr Asn Tyr Val Thr Tyr Lys  
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Tyr Gly Gly Gly Gly Glu Tyr Met Ser Gln Thr Gly Phe Gly Trp Ser  
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10 Asn Gly Val Val Leu Ala Leu Leu Glu Glu Phe Gly Trp Pro Glu Asp  
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Leu Lys Ile Asp Cys  
580

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## (2) INFORMATION FOR SEQ ID NO: 11:

- 20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 25 (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: YES
- 30 (ix) FEATURE:  
(A) NAME/KEY: modified\_base  
(B) LOCATION: 6  
(D) OTHER INFORMATION: /mod\_base= i
- 35 (ix) FEATURE:  
(A) NAME/KEY: modified\_base  
(B) LOCATION: 15  
(D) OTHER INFORMATION: /mod\_base= i
- 40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGYGGNMGMT TYRWNGARKT MTAYKRYTGG GAC

33

## (2) INFORMATION FOR SEQ ID NO: 12:

- 45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
50 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: YES

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## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 3
- (D) OTHER INFORMATION: /mod\_base= i

5

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 6
- (D) OTHER INFORMATION: /mod\_base= i

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## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 9
- (D) OTHER INFORMATION: /mod\_base= i

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- (A) NAME/KEY: modified\_base
- (B) LOCATION: 12
- (D) OTHER INFORMATION: /mod\_base= i

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## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 15
- (D) OTHER INFORMATION: /mod\_base= i

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## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 21
- (D) OTHER INFORMATION: /mod\_base= i

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTNCCNGGNG GNCGNTTYRW NGARKT

35

26

## (2) INFORMATION FOR SEQ ID NO: 13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

## (ii) MOLECULE TYPE: DNA (genomic)

45

## (iii) HYPOTHETICAL: YES

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 3
- (D) OTHER INFORMATION: /mod\_base= i

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41

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 9
- (D) OTHER INFORMATION: /mod\_base= i

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## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 12
- (D) OTHER INFORMATION: /mod\_base= i

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## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 15
- (D) OTHER INFORMATION: /mod\_base= i

15

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 18
- (D) OTHER INFORMATION: /mod\_base= i

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGNGGYTGNS WNCGNYRNAG RTARTA

26

25

## (2) INFORMATION FOR SEQ ID NO: 14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

## (ii) MOLECULE TYPE: DNA (genomic)

35

## (iii) HYPOTHETICAL: YES

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /mod\_base= i

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## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 7
- (D) OTHER INFORMATION: /mod\_base= i

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## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: 19
- (D) OTHER INFORMATION: /mod\_base= i

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42

(ix) FEATURE:

(A) NAME/KEY: modified\_base

(B) LOCATION: 22

(D) OTHER INFORMATION: /mod\_base= i

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

NSCRTTNRYC CATCCRAANC CNTC

10

24

## CLAIMS

1. A process for producing trehalose in plant cells capable of producing trehalase by growing plant cells having the genetic information required for the production of trehalose and trehalase, or cultivating a plant or a part thereof comprising such plant cells, characterised in that said plant cells are grown, or said plant or a part thereof, is cultivated in the presence of a trehalase inhibitor.
2. A process according to claim 1, wherein said plant cells have been genetically altered so as to contain a chimeric trehalose phosphate synthase gene in a plant expressible form, preferably wherein the trehalose phosphate synthase gene comprises an open reading frame encoding trehalose phosphate synthase from *E. coli* in plant expressible form, more preferably wherein the open reading frame encoding trehalose phosphate synthase from *E. coli* is downstream of the CaMV 35S RNA promoter or the potato patatin promoter.
3. A process according to claim 1 or 2, wherein a *Solanum tuberosum* plant is cultivated, preferably wherein said plant has micro-tubers.
4. A process according to claim 3, wherein said plant is cultivated *in vitro*.
5. A process according to any one of claims 1 to 4, wherein said trehalase inhibitor comprises validamycin A in a form suitable for uptake by said plant cells, said plant, or a part thereof, preferably wherein the concentration of validamycin A is between 100 nM and 10 mM, more preferably between 0.1 and 1 mM, in aqueous solution.
6. A process according to any one of claims 1 to 4, wherein said trehalase inhibitor comprises the 86kD protein of the cockroach (*Periplaneta americana*) in a form suitable for uptake by said plant cells, said plant, or a part thereof.

7. A process according to any one of claims 1 to 4, wherein said plant cells have been genetically altered to contain the genetic information for a trehalase inhibitor, preferably wherein the trehalase inhibitor is the antisense gene to the gene encoding the information for trehalase or wherein the trehalase inhibitor is the 86kD protein of the American cockroach (*Periplaneta americana*).
8. A process according to any one of claims 1 to 7, wherein a plant, or a part thereof, accumulates trehalose in an amount above 0.01 % (fresh weight).
9. A plant, or a part thereof, or plant cells, obtainable by a process according to any one of the claims 1 to 8, which contain trehalose in an amount above 0.01% (fresh weight), preferably wherein said plant, or a part thereof is a *Solanaceae* species, more preferably *Solanum tuberosum* or *Nicotiana tabacum*.
10. A plant part according to claim 9, which is a tuber or a micro-tuber.
11. Tuber or micro-tubers of *Solanum tuberosum* containing trehalose.
12. Use of a plant, or plant part, according to claim 9 for extracting trehalose.
13. Use of a plant, or plant part, according to claim 9 in a process of forced extraction of water from said plant or plant part.
14. A plant according to claim 9, which has an increased stress tolerance, preferably increased drought tolerance.
15. A chimaeric plant expressible gene comprising in sequence a transcription initiation region obtainable from a gene, preferentially expressed in a plant part, particularly the patatin gene from *Solanum tuberosum*, a 5'-untranslated leader, an open reading frame encoding a trehalose phosphate synthase activity, and downstream of said open



reading frame a transcriptional terminator region, preferably wherein said transcriptional terminator region is obtainable from the proteinase inhibitor-II gene of *Solanum tuberosum*.

5 16. A vector comprising a chimaeric plant expressible gene according to claim 15.

17. A recombinant plant genome comprising a chimaeric gene according to claim 16.

10

18. A plant cell having a recombinant genome according to claim 17.

19. A plant or a part thereof, consisting essentially of cells according to claim 18, wherein said plant is *Solanum tuberosum*.

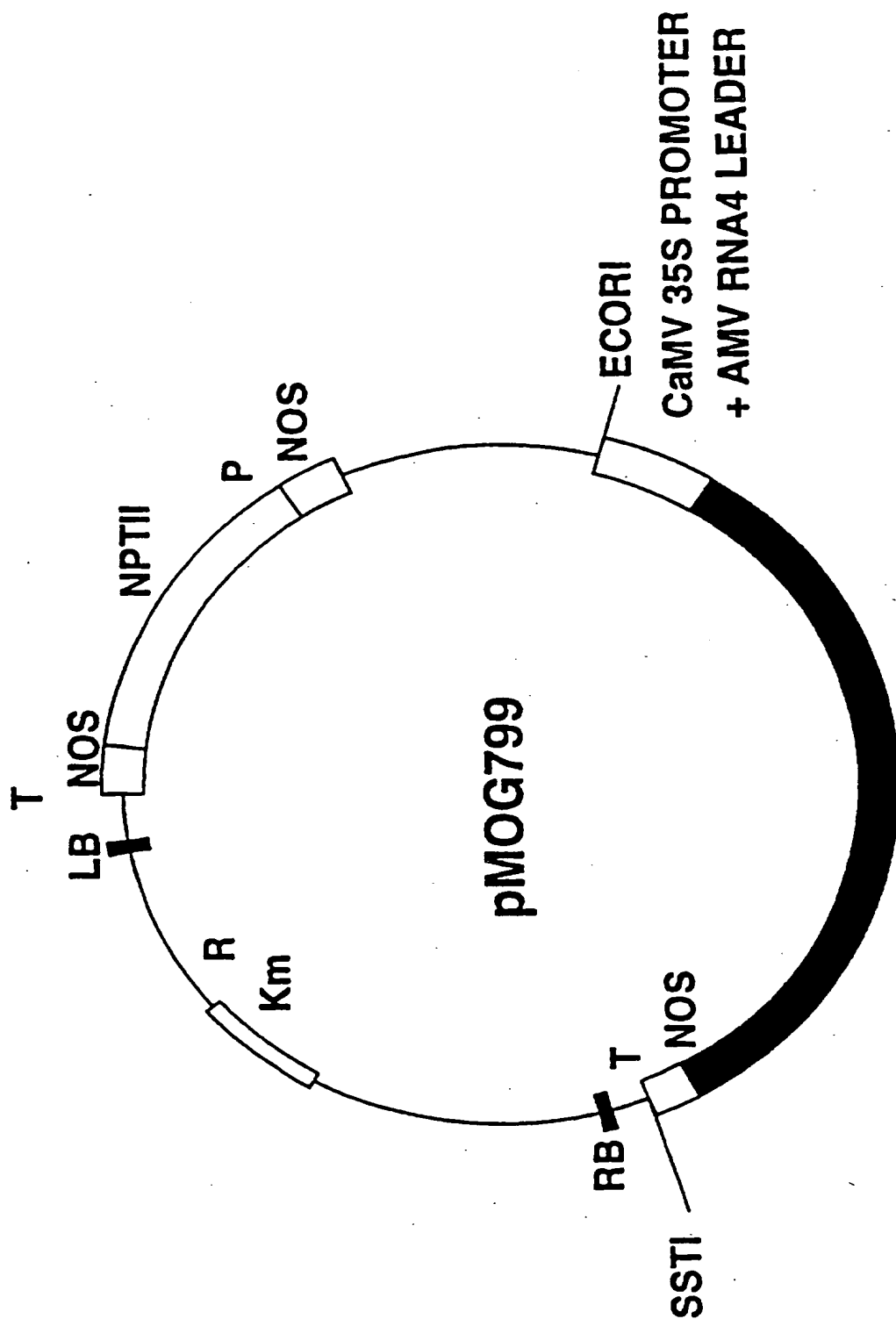
15

20. A plant part according to claim 19, which is a tuber or a micro-tuber.

21. A process for obtaining trehalose, comprising the steps of growing  
20 plant cells according to claim 18, or cultivating a plant according to claim 19, or cultivating a plant part according to any one of claims 19 or 20, extracting trehalose from said plant cells, plants or parts.

22. A process for obtaining trehalose, comprising the steps of  
25 producing trehalose in plant cells, a plant or a part thereof, according to a process of any one of claims 1 to 8, and separating or extracting trehalose from said plant cells, plant or part thereof.

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TREHALOSE-6-PHOSPHATE SYNTHASE

Figure 1

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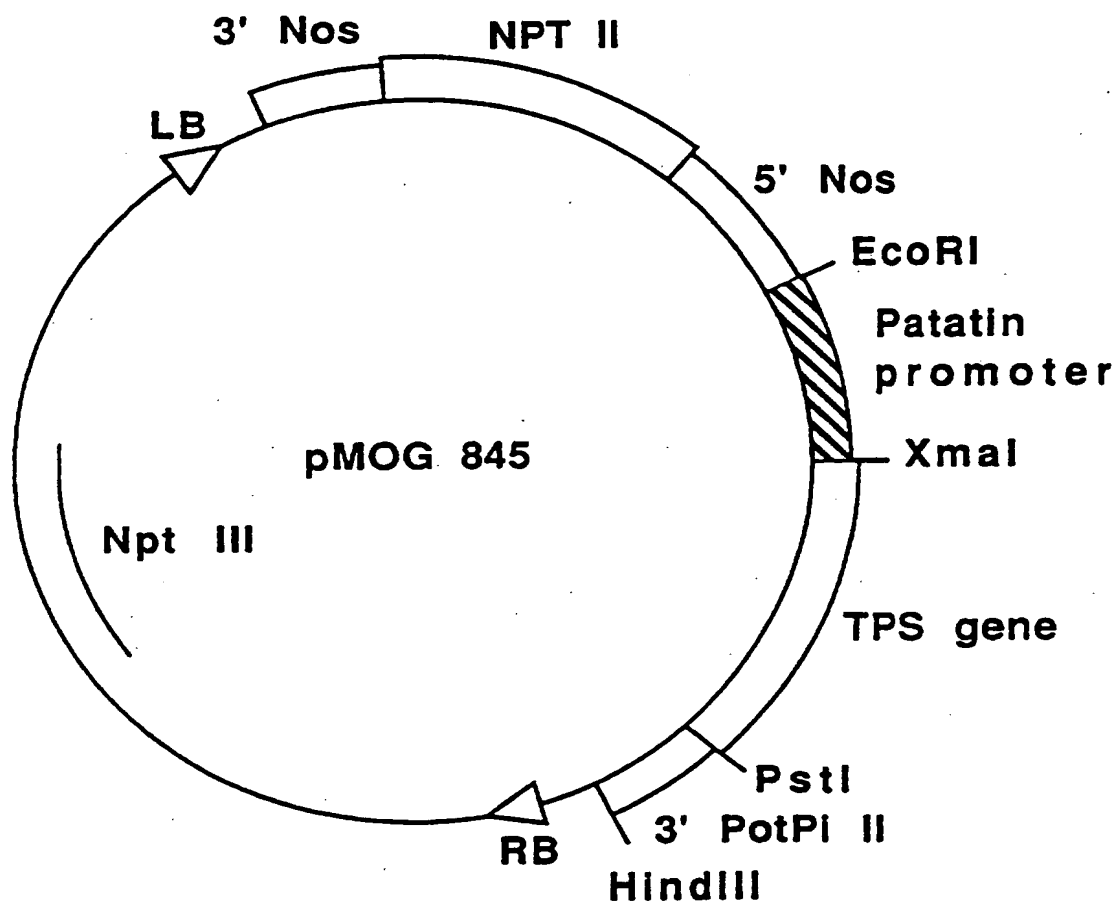


Figure 2

3 / 4

# ENGINEERING OF TREHALOSE-PRODUCTION IN PLANTS

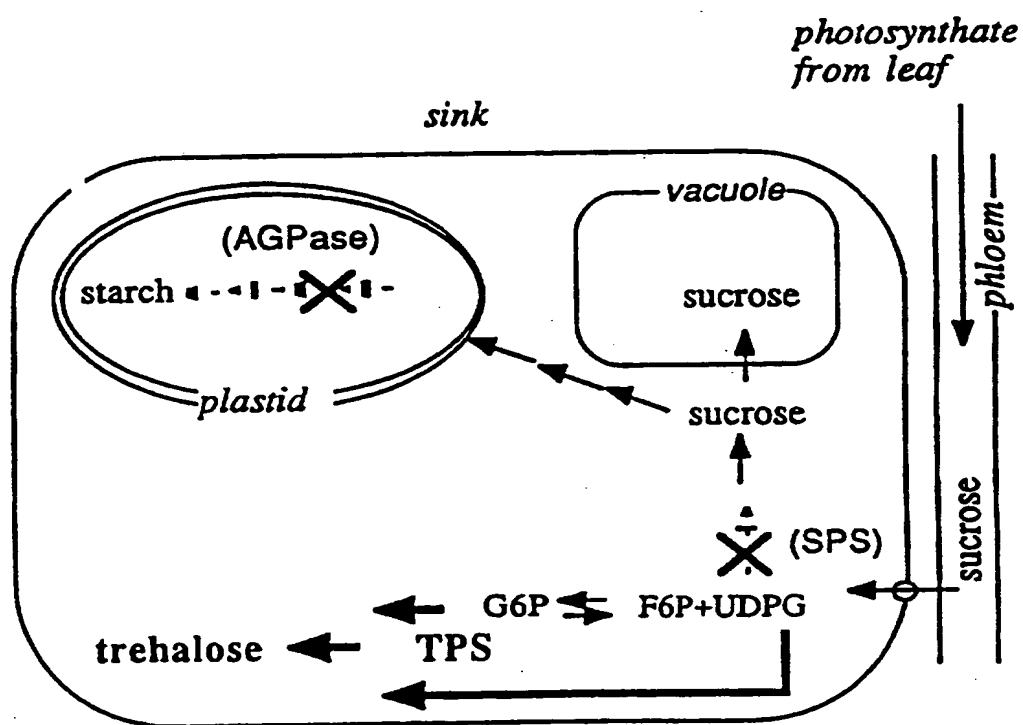


Figure 3

**4 / 4**

[illegible]

### Figure 4

BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

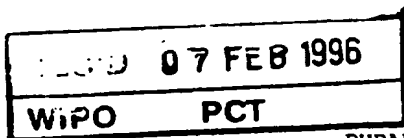
INTERNATIONAL FORM

Mogen International N.V. Einsteinweg 97 2333 CB LEIDEN Nederland
<i>name and address of depositor</i>

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:  E. coli strain SOLR tm pMOG1026	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  CBS 804.95
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by:  <input checked="" type="checkbox"/> a scientific description  <input type="checkbox"/> a proposed taxonomic designation  (mark with a cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary accepts the microorganism identified under I above, which was received by it on Friday, 8 December 1995 (date of the original deposit) <sup>1</sup>	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depositary Authority on not applicable (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on not applicable (date of receipt of request for conversion)	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name: Centraalbureau voor Schimmelcultures  Address: Oosterstraat 1 P.O. Box 273 3740 AG BAARN The Netherlands	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  M.C. Agterberg drs F.M. van Asma dr M.C. Agterberg  Date: Monday, 8 January 1996

<sup>1</sup> Where Rule 6.4(d) applies, such date is the date on which the status of international  
depository authority was acquired.



EP/A/II/12  
page 24

BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Mogen International N.V.  
Einsteinweg 97  
2333 CB LEIDEN  
Nederland

*name and address of the party to whom the  
viability statement is issued*


VIABILITY STATEMENT  
issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Mogen International N.V.  Address: Einsteinweg 97 2333 CB LEIDEN Nederland	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  CBS 804.95  Date of the deposit or of the transfer: <sup>1</sup>  Friday, 8 December 1995
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on Thursday, 12 January 1995 <sup>2</sup> . On that date, the said microorganism was  <input checked="checked" type="checkbox"/> <sup>3</sup> viable  <input type="checkbox"/> <sup>3</sup> no longer viable	

<sup>1</sup> Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

<sup>2</sup> In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

<sup>3</sup> Mark with a cross the applicable box.

<b>IV. CONDITIONS UNDER WHICH THE VIABILITY HAS BEEN PERFORMED<sup>4</sup></b>	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
<b>Name:</b> Centraalbureau voor Schimmelcultures	<b>Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):</b>
<b>Address:</b> Oosterstraat 1 P.O. Box 273 3740 AG BAARN The Netherlands	<div style="text-align: right;">drs F.M. van Asma dr M.C. Agterberg</div> <div style="text-align: center;"></div> <b>Date:</b> Monday, 8 January 1996

<sup>4</sup> Fill in if the information has been requested and if the results of the test were negative.



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 96/00080

A. CLASSIFICATION OF SUBJECT MATTER		
IPC 6	C12N15/82 C12N5/04	C12N15/54 A23L3/3562
C12N15/56 A01H5/00	C12N15/12	C12P19/12
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 6 C12N C12P A01H A23L		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEM. SYSTEM. ECOL., vol. 18, no. 2/3, 1990, pages 107-110, XP000567557 ADAMS, R.P., ET AL.: "COMPARISON OF FREE SUGARS IN GROWING AND DESSICATED PLANTS OF SELAGINELLA LEPIDOPHYLLA" see the whole document ---	9,12,13
X	COMPTES RENDUES ACAD. SC. PARIS, vol. 259, - 20 July 1964 pages 635-637, XP002000476 QUILLET, M., ET AL.: "SUR L'ACCUMULATION CONCOMINANTE DU SACCHAROSE ET DU TRAHALOSE CHEZ PLUSIEURS ESPECES DE SELAGINELLES INDIGENES ET EXOTIQUES" see the whole document ---	9,14
-/--		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
15 April 1996		23.04.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040; Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016		Authorized officer  Maddox, A

## INTERNATIONAL SEARCH REPORT

 International Application No  
 PCT/EP 96/00080

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PLANT PHYSIOLOGY SUPPLEMENT, vol. 108, no. 2, June 1995, page 149 XP002000477 GODDIJN, O.J.M., ET AL.: "Transgenic tobacco plants as a model-system for the production of trehalose" see abstract 786	9
P,X	--- WO,A,95 01446 (MOGEN INT ;HOEKEMA ANDREAS (NL); PEN JAN (NL); DOES MIRJAM PETRONE) 12 January 1995 see page 13, line 3 - line 10	1-4,7
P,X	--- WO,A,95 06126 (MOGEN INT ;HOEKEMA ANDREAS (NL); PEN JAN (NL); DOES MIRJAM PETRONE) 2 March 1995 see page 14, line 6 - line 15	1-4,7
A	--- EP,A,0 341 885 (ICI PLC) 15 November 1989 see claims 1,3	1
A	--- PLANT PHYSIOL (BETHESDA) 68 (6). 1981 (RECD. 1982). 1369-1374., XP000567527 VELUTHAMBI K: "TREHALOSE TOXICITY IN CUSCUTA-REFLEXA CORRELATION WITH LOW TREHALASE ACTIVITY" see the whole document	1
A	--- PHYTOCHEMISTRY (OXF) 29 (8). 1990. 2525-2528., XP002000478 KENDALL E J: "TREHALASE ACTIVITY IN PLANT TISSUE CULTURES." see the whole document	1
A	--- CURR BIOL 2 (11). 1992. 594-596., XP002000479 TOMOS D: "RESURRECTION PLANTS LIFE WITHOUT WATER." see page 596, left-hand column, last paragraph	9,14
A	--- WO,A,93 17093 (ALKO AB OY) 2 September 1993 see the whole document	1-22
A	--- EP,A,0 451 896 (GIST BROCADES NV) 16 October 1991 see the whole document	1-22
A	--- BIOTECHNOLOGY, vol. 12, pages 1328-1329, XP002000480 KIDD, G., ET AL.: "TREHALOSE IS A SWEET TARGET FOR AGBIOTECH" see the whole document	1-22

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 96/00080

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 577 915 (ALGIST BRUGGEMAN NV) 12 January 1994 see the whole document ---	1-22
E	WO,A,96 00789 (ALKO GROUP LIMITED ;LONDESBOROUGH JOHN (FI); TUNNELA OUTI (FI); HO) 11 January 1996 see page 25; table 1 -----	9,12,14

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 96/00080

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
WO-A-9501446	12-01-95	WO-A-	9506126	02-03-95
		AU-B-	7384694	24-01-95
		CA-A-	2166063	12-01-95
		FI-A-	956317	29-12-95
		NO-A-	955354	02-01-96
		AU-B-	4953393	21-03-95
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WO-A-9506126	02-03-95	AU-B-	4953393	21-03-95
		AU-B-	7384694	24-01-95
		CA-A-	2166063	12-01-95
		WO-A-	9501446	12-01-95
		FI-A-	956317	29-12-95
		NO-A-	955354	02-01-96
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EP-A-0341885	15-11-89	AT-T-	126659	15-09-95
		AU-B-	628818	24-09-92
		AU-B-	3469789	16-11-89
		CA-A-	1331837	06-09-94
		DE-D-	68923903	28-09-95
		DE-T-	68923903	04-04-96
		JP-A-	2031625	01-02-90
		US-A-	5387757	07-02-95
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WO-A-9317093	02-09-93	US-A-	5422254	06-06-95
		AU-B-	3500993	13-09-93
		CA-A-	2130082	02-09-93
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